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STUDIES ON THE HEMOGLOBIN OF ANADARA INFLATA (REEVE)

I. PURIFICATION AND PROPERTIES OF THE CRYSTALLINE HEMOGLOBIN*

By YASUO YAGI,** TOSHIO MISHIMA, TSUNEKO TSUJIMURA, KIMIKO SATO AND FUJIO EGAMI

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(Received for publication, July 5, 1956)

A number of studies have been carried out on the structure of several vertebrate hemoglobins derived from a variety of species in crystalline form. However, the same type of respiratory pigments from invertebrates, designated originally as erythrocruorins and recently proposed by Keilin and Hartree (*2*) to align them with other vertebrate hemoglobins in nomenclature, has been taken up only in a few cases (*3, 4*) as subjects of such chemical studies, because of difficulties obtaining sufficient amounts of materials with required high degree of purity.

Recently, we have succeeded in obtaining crystalline preparation of hemoglobin from erythrocytes of a species of *Lamellibranchia*, *Anadara* (= *Arca*) *inflata* (Reeve), which is easily available on the market in Japan. The method for its purification and some physical and chemical properties of the purified material will be described in this report.

EXPERIMENTAL

Purification of Anadara-Hemoglobin—Hemolymph of *Anadara inflata* was collected by heart puncture after breaking a part of the shell with a small hammer. (About 1,300 ml. of hemolymph were obtained from *ca.* 80 pieces of fresh animals weighing 7.5 kg.). The red hemolymph was repeatedly filtered through a gauze-layer to remove small shell fragments and fine mud particles and centrifuged (1,240 RCF, 20 minutes). The precipitated red erythrocytes were washed four times with 3 per cent sodium chloride

* A part of this work was presented at the meeting of " Société franco-japonaise de biologie " at Nagoya in December, 1955 (*1*).

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solution by centrifugation, suspended in the same sodium chloride solution, then subjected to gentle aeration for one hour (oxygenation). The hemolysis was carried out by repeating freezing and thawing procedures with dry ice. Since *Anadara*-hemoglobin is labile and easily denatured, all the procedures hereafter were carried out in a cold room (+5°).

The supernatant solution after removal of stromata by high speed centrifugation (3,550 RCF, 30 minutes), was shown to contain at least three components by moving boundary electrophoresis (Tiselius apparatus), as well as by its salting-out curve according to Derrien (*5*). For the fractionation, an equimolar mixture of potassium phosphate (1.75 *M* KH_2PO_4+1.75 *M* K_2HPO_4, pH 6.7) was stocked in the cold room (+5°). A part of salts was crystallized as a deposit and the clear supernatant solution (pH 6.2), designated as " cold phosphate mixture " in the following description, was used throughout the experiments. The cold phosphate mixture was added dropwise to the red supernatant to make a final concentration of 52 per cent (*v/v*). After standing 2 hours at +5°, the mixture was centrifuged and the red precipitate was discarded. The phosphate concentration of the dark red supernatant was raised to 66 per cent and the resulted precipitate was collected after 2 hours standing. In this case, the precipitate floated as a surface layer over the clear red solution by centrifugation. The precipitate was suspended in a solution containing 66 per cent of cold phosphate mixture, then diluted to 52 per cent with distilled water. Hyflo Super-cel was added and a small amount of insoluble material was filtered off. The phosphate concentration of the filtrate was raised to 66 per cent and the precipitate was collected by centrifugation as before. It was dissolved in a small volume of water and dialyzed against 0.01 *M* phosphate buffer (pH 7.0). This amorphous preparation was found to be homogeneous by electrophoresis, by ultracentrifugal analysis, and by its salting-out curve. About 800 mg. were obtained.

For crystallization, the final precipitate was dissolved in a solution containing 50 per cent cold phosphate mixture. A concentration of about 5 mg. protein per ml. was found to be most suitable for crystallization. The solution was filtered and the cold phosphate mixture was added dropwise until a very faint turbidity appeared (usually *ca.* 65 per cent for the above protein concentration). The turbidity disappeared when the solution was dipped into crushed ice. The solution kept in crushed ice was brought out from the cold room and let it stand over-night, as it is, in a room at 13–15°. Crystals were collected on a glass filter and stored in a refrigerator. About 300 mg. crystals were obtained from 7.5 kg. of fresh animal.

An example of crystalline preparations is shown in Fig. 1(a) and a typical crystal structure in Fig. 1(b). The crystal seems to belong to rhombic system upon examination under the polarizing microscope.

Electrophoresis—The moving boundary electrophoresis was carried out with an apparatus manufactured by Hitachi Manufacturing Co., Ltd. (diagonal cylindrical lens system).

Salting-out Curve—The salting-out curve was determined according to Derrien (*5*) with an equimolar mixture of potassium phosphate (1.75 *M* KH_2PO_4+1.75 *M*

K_2HPO_4, pH 6.7). The final concentration was about 0.5–1.0 mg. protein/ml. The optical density of each filtrate at each salt concentration was measured with a Beckman Spectrophotometer (Model DU) at 275 mμ and at 576 mμ and plotted against salt concentration.

Absorption Spectra—The absorption curves of several hemoglobin derivatives were

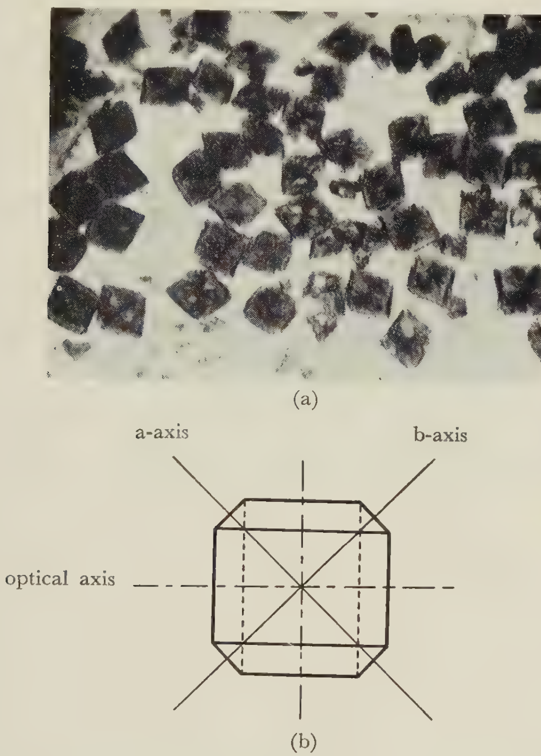

FIG. 1. (a) Crystalline preparation (×150). (Kindly photogrphed by Mr. Kenzo Takata.)
(b) Crystal structure.

determined with a Beckman Spectrophotometer. The conversion of oxyhemoglobin to other derivatives was carried out by usual procedures.

Determination of Fe—The Fe content was determined with salt-free preparations by a colorimetric method with *o*-phenanthroline after mineralization with a mixture of sulfuric acid and perchloric acid (*6*, *7*).

Determination of Histidine—The histidine content was determined by a modified

Knoop's procedure (*8*) after hydrolysis with 6 *N* HCl at 120° for 15 hours in a sealed tube. A crystalline preparation of horse oxyhemoglobin was analyzed by the same procedure and the analytical data were found to be in good agreement with those reported by other researchers.

RESULTS AND DISCUSSION

Physical Properties—Examination of the purified preparations revealed that even the amorphous is essentially homogeneous as determined by electrophoresis (Fig. 2), by ultracentrifugation (*9*) and by salting-out curve. The physical constants were summarized in Table I.

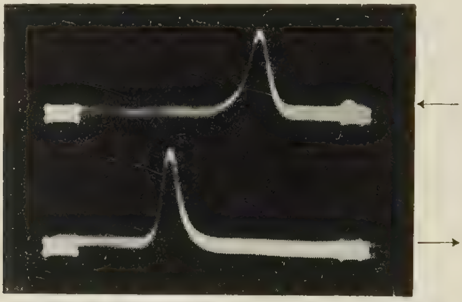

FIG. 2. Electrophoretic pattern obtained of *Anadara*-hemoglobin (1.07 per cent protein) in Veronal buffer of pH 8.2_3 with an ionic strength of 0.1 at 3.4°, after electrophoresis for 10800 sec. at a field strength of 3.542 volts/cm. The upper was " Descending boundary " and the lower " Ascending boundary."

As reported in the part of Appendix, the sample solution showed a single peak between pH 6.1 and 8.1 in the ultracentrifugal pattern. From the sedimentation constant, the diffusion constant at pH 7.45, and the supposed value of 0.75 for the partial specific volume, it was derived that the *Anadara*-hemoglobin has a molecular weight of *ca.* 73,000 with f/f_0 1.25. As compared with the value (mol. wt.=34,500) obtained by Svedberg (*10*) on the erythrocruorin from *Anadara* (*Arca*) *pexata*, this is twice as large and rather similar to the molecular weight of vertebrate hemoglobins.

The purified preparation behaved as a single component by moving boundary electrophoresis between pH 6.5 and 8.6. The electrophoretic mobility at pH 6.5 and at pH 8.2 was found to be identical within the experimental error. Considering the fact that the electrophoretic mo-

bility of a protein depends upon the degree of its dissociation at the pH where the measurement is carried out and the dissociation curve of a protein often shows a broad shoulder, it would not be unreasonable that these two values could not be distinguished within the limit of our experimental precision. The electrophoresis at lower pH could not be performed because of the instability of *Anadara*-hemoglobin in acidic medium, but it is quite evident that the isoelectric point exists at a pH lower than 6 as Svedberg and Ericksson-Quensel (*10*) had indicated.

Anadara-hemoglobin is much more unstable than the horse hemoglobin and easily denatured in acidic medium lower than pH 6, by electrodialysis or by contact with organic solvents, ethanol, ether, or toluol.

Chemical Properties—The analytical results of the purified material were summarized in Table I. The Fe content of the crystalline pre-

TABLE I

Physical and Chemical Properties

Electrophoretic mobility		-3.1×10^{-5} cm.^{2}/sec. volt (pH 8.2_3, Veronal buffer, $\mu=0.1$)
(3.4°)		-3.1×10^{-5} cm.^{2}/sec. volt (pH 6.5_5, Phosphate buffer, $\mu=0.1$)
Sedimentation constant	$s_{20,\,w}$	4.5_7 S (pH 7.4_5)
Diffusion constant	$D_{20,\,w}$	6.1×10^{-7} cm.^{2}/sec. (pH 7.4_5)
Molecular weight		73,000
	f/f_0	1.25
Nitrogen	%	15.7
Iron	%	0.29
Histidine	%	2.3

paration was found to be 0.29 per cent. The minimum molecular weight based on its Fe content is 19,100 hence the *Anadara*-hemoglobin contains 4 atoms of iron, *i.e.* 4 molecules of heme per molecule.

The histidine residues are said to play an important role in the combination of protoheme with the protein component in some vertebrate hemoglobins, and it is well known that this amino acid is abundant in vertebrate hemoglobins, but much poorer in invertebrate respiratory pigments including erythrocruorins (invertebrate hemoglobins), chloro-

TABLE II

Position of the Maximum Absorption and Their Intensity

	Anadara-hemoglobin		Horse-hemoglobin (12)	
	Position (mμ)	Intensity*	Position (mμ)	Intensity*
Oxyhemoglobin**	575 (α) 540 (β) 412 (γ)	12.7 13.2 120	576 (α) 541 (β) 414 (γ)	16.1 15.3 132
Hemoglobin (reduced)	555	13.5	556	13.5
Carboxyhemoglobin	570 540	12.1 11.7	570 540	14.4 15.7
CN-methemoglobin	539–547	10.1	540	11.4
Globin-hemochrome (alkali-denatured)	557 528	27.0 11.5	559 528	30.6 14.5
Pyridine-hemochrome	555 525	29.9 13.0	557 525	32.2 16.8

* Expressed in terms of their extinction coefficients per mg. atom Fe.

** Measured at pH 6.0. No shift of positions of these peaks was observed by varying the pH to 4.0 or 10.0.

cruorins and hemerythrins. The value obtained for *Anadara*-hemoglobin was considerably lower than those for vertebrate hemoglobins and rather similar to the chlorocruorin preparation from *Spirographis* (*3*, *4*). This fact, in combination with its rather acidic isoelectric point, shows that the protein component is entirely different from those of vertebrate hemoglobins.

Absorption Spectra of Some Derivatives—The absorption spectra of the oxyhemoglobin preparation and several derivatives from it were determined by a Beckman Spectrophotometer. The positions of maximum absorption and their intensity were collected in Table II. The positions of maximum absorption were essentially the same as those found for vertebrate hemoglobin derivatives, showing that their prosthetic groups are very similar (*11*). However, their extinction coefficients per milligram atom Fe were slightly lower than those of vertebrate hemoglobin derivatives (*12*).

SUMMARY

1. Methods for purification of the hemoglobin of *Anadara inflata* (Reeve) in homogeneous state and for its crystallization were described.

2. The purified preparation was examined by moving boundary electrophoresis, by ultracentrifugal analysis and by salting-out curve method, and was found to be homogeneous on all these criteria.

3. Based on the sedimentation constant and the diffusion constant, a molecular weight of *ca.* 73,000 was derived. Minimum molecular weight based on its Fe content was found to be *ca.* 19,100, hence the *Anadara*-hemoglobin molecule contains 4 molecules of heme per molecule.

4. The identity of the prosthetic group with those of vertebrate hemoglobins was supported by the absorption spectra of several derivatives.

5. The difference of the protein component from those of vertebrate hemoglobins was shown by its low histidine content and by its electrophoretic behavior.

We wish to thank Prof. Tadao Sato for his deep interest and useful suggestions and Dr. Keinosuke Nagasawa for examination of the crystall structure.

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APPENDIX: ON THE MOLECULAR WEIGHT OF HEMOGLOBIN OF ANADARA INFLATA (REEVE)

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(Received for publication, July 5, 1956)

The sample of hemoglobin of *Anadara inflata* (Reeve) (*1*), supplied by Dr. Yagi, was examined in a Spinco model E ultracentrifuge and a Neurath-type diffusion cell (*2*) equipped with a Svensson's schlieren optical system (*3*). To prevent denaturation of hemoglobin, measurements were performed at temperatures below 15°.

Ultracentrifugal patterns of this sample in buffer solutions of pH 5.9_8, 7.4_5 and 8.1_5 at ionic strength of 0.1 showed always one symmetrical boundary indicating its homogeneity. A representative pattern is given in Fig. 1. The sedimentation coefficient reduced to the value in water

FIG. 1. Sedimentation pattern of hemoglobin of *Anadara inflata* (Reeve) about 90 minutes after reaching the speed of 59,780 r.p.m. Solvent: phosphate-NaCl buffer of pH 7.4_5 and ionic strength of 0.1. Concentration: 1.07 per cent. Bar angle: 65°.

at 20° was found to be independent of the pH value and the hemoglobin concentration, as is shown in Table I. The sedimentation constant at infinite dilution was extrapolated to 4.5_7 S (Svedberg unit) at pH 7.4_5.

The diffusion pattern at 15° of 0.5_4 per cent hemoglobin solution in a phosphate-NaCl buffer of pH 7.4_5 and ionic strength of 0.1 showed

TABLE I

Sedimentation Coefficient of Hemoglobin of Anadara inflata

Buffer	pH	Concn.	Temp. of measurements	$s_{20,w}$
Phosphate-NaCl	7.4_5	1.07%	7.6	4.4_8 S
”	”	0.54	5.1	4.4_4
”	”	0.21	8.0	4.5_9
”	5.9_8	1.07	9.2	4.4_1
Veronal-NaCl	8.1_5	”	11.7	4.2_4

a Gaussian curve. Thus the solution seemed to be homogeneous and the diffusion coefficient free of concentration dependency. The diffusion coefficient calculated from second moments of the refractive index-gradient curve (*4*) was 6.1×10^{-7} cm.^{2}/sec. at 20° in water. In the following calculation, this value was used as the diffusion constant at infinite dilution.

Combining the values of sedimentation and diffusion constants and assuming a value of 0.75 for the partial specific volume, the molecular weight (non-hydrated) of this hemoglobin was calculated to be 73,000. This value, and the value of frictional ratio, 1.25, coincide fairly well with those of vertebrate hemoglobins (*5*).

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STUDIES ON THE HEMOGLOBIN OF ANADARA INFLATA (REEVE)

II. TERMINAL AMINO ACID RESIDUES*

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(Received for publication, July 5, 1956)

The terminal structures of hemoglobins from several species of mammals have been much studied since the original works by Porter and Sanger (*2*). Recently, Ozawa and Satake (*3*) extended their studies to lower classes of vertebrates and showed that all the hemoglobins examined so far (horse, pig, dog, cat, rabbit, guinea pig, rat, chicken and snake) have valine residues at the *N*-terminal positions, although those from ruminants (bovine, sheep and goat) have methionine residues in addition to valine. The variety of C-terminal amino acids of mammal hemoglobins also seemed to be rather limited. Glutamine residue was the only one found in cases of ruminants and glutamine plus glycine or alanine were detected from those of other mammals (*4*).

As reported in the previous part of this article (*5*), we have obtained an invertebrate hemoglobin (erythrocruorin) in homogeneous state from a species of *Lamellibranchia*, *Anadara inflata* (Reeve) and showed the difference of its protein component from those of vertebrate hemoglobins. In order to confirm this point furthermore and to get some informations about the structures of invertebrate hemoglobins, on which very few studies have been made from such point of view, we have tried in this work to elucidate the *N*- and C-terminal amino acids of the purified *Anadara*-hemoglobin. The results obtained indicated that the protein component of *Anadara*-hemoglobin has entirely different amino acid residues at the ends of its polypeptide chains, compared with those for vertebrate hemoglobins.

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* A part of this work was presented at the meeting of " Société franco-japonaise de biologie " at Nagoya in December, 1955 (*1*).

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EXPERIMENTAL

Materials

Anadara-Hemoglobin—This was prepared according to the method described in our previous report (*5*). Both the crystalline and the amorphous material were used, but the results showed essentially no difference. For the identification of C-terminal amino acids by carboxypeptidase, the soluble crystalline preparation and the only partly soluble lyophilized material were both used with the same results.

Protein Component of Anadara-hemoglobin—The hemoglobin solution was mixed with the same volume of 0.1 *N* HCl, then with 20 volumes of ice-cold acetone containing 1/10 volume of *N* HCl according to the method of Anson (*6*). The faintly gray precipitate (protein component) was washed three times with cold acetone, then dissolved in water. The solution was directly used as samples for identification of *N*-terminal amino acids. When the acetone precipitate was once dried in vacuo, a large part of the protein component was denatured and became almost insoluble in water.

Crystalline Carboxypeptidase—A commercial crystalline carboxypeptidase from Worthington Biochemical Sales Co., N. J., U.S.A. (3 times recrystallized) was used throughout the experiments. It was dialyzed for one day in a refrigerator before use.

Diisopropylfluorophosphate—Synthesized by us according to Saunders and Stacey (*7*).

Anhydrous Hydrazine—Commercial hydrazine hydrate containing ca. 80 per cent hydrazine was dehydrated according to Satake[1]. The water content was less than 1 per cent as revealed by titration with potassium bromate.

Phenylisothiocyanate—Prepared by us according to Dains, Brewster, and Olander (*8*).

2,4-Dinitrofluorobenzene—A commercial product.

METHODS

Determination of C-terminal Amino Acids—Two independent methods were applied, one by carboxypeptidase digestion and the other by hydrazinolysis according to Akabori and Ohno.

(*I*) *Method by Carboxypeptidase Digestio—*

n) *Carboxypeptidase Digestion*: The carboxypeptidase (CPD) solution (63 μg.) in 0.5 ml. borate buffer of pH 8.1[2] was incubated at 37° with 0.01 ml. of 1 per cent diisopropylfluorophosphate (DFP) in isopropanol for 15 minutes to inactivate endopeptidases which might exist in the enzyme preparation as possible contaminants (CPD:DFP= 1:50 (mole ratio)). After incubation, a solution of crystalline hemoglobin or a suspension of partly soluble lyophilized preparation (6.2 mg. in 2.0 ml. borate buffer)

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1) Personal communication.

2) 0.2 *M* H_3BO_3+0.05 *M* NaCl soln. 70 ml.+0.05 *M* $Na_2B_4O_7\cdot 10H_2O$ soln. 30 ml.

was added and incubated for a definite time (CPD:Hb=1:50). The red clear solution increased brownish color slowly and became turbid, giving a brownish uniform suspension[3] after 4 hours incubation. The pH of the solution remained around 8.0 even after 8 hours incubation.

b) *Separation of Amino Acids liberated during the Digestion* (*9, 10*): After digestion, the pH was lowered to 3.0 to stop the enzyme action. 10 minutes later, the pH was brought back to 5.4–5.6 to remove most part of proteins in the form of precipitate. The amino acids liberated during the digestion were removed from the supernate by shaking for one hour with 100 mg. Dowex 50X2, (20–50 mesh, H^+-form) at pH 3.0 and the supernatant solution containing the remaining protein was discarded. The resin was washed with distilled water, then shaken with water for one hour. The amino acids absorbed on the resin was finally eluted by shaking with 1 ml. of 5 *M* ammonium hydroxide for 15 minutes. The eluate and the washing liquids were concentrated in vacuo under 40° and the residue was taken in a definite volume of distilled water, then submitted to paper chromatography. As tested with the known amounts of amino acids, their recovery was almost quantitative by this procedure.

c) *Paper Chromatography and Determination of Amino Acids*: One dimensional chromatography with *n*-butanol-acetic acid-water (4:1:5) (descending technique) and two dimensional chromatography with methanol-water-pyridine (80:20:4) and tert.-butanol-methylethylketone-water-diethylamine[4] (40:40:20:4) (both ascending) (*11*) were used for qualitative identification of amino acids. The position of spots was determined by spraying 0.1 per cent solution of ninhydrine in a mixture of ethanol (50 ml.), acetic acid (15 ml.) and collidine (2 ml.) on the paper.

For quantitative analyses, one dimensional chromatography with butanol-acetic acid-water (descending) was used. The paper was dried by standing at room temperature, then at 100° for 15 minutes. The position of spots was determined under ultraviolet lamp. Each spot was cut out from the paper and the amount of each amino acid was determined according to the method of Fowden (*12*).

(*II*) *Method by Hydrazinolysis*—

Hydrazinolysis was carried out according to Akabori and Ohno (*13*). This method was used only for qualitative identification of C-terminal amino acid residues which are sometimes ambiguous by the former method.

a) *Hydrazinolysis and Dinitrophenylation*: 30 mg. of lyophilized hemoglobin preparation was heated with 0.5 ml. of anhydrous hydrazine at 100° for 10 hours taking care of avoiding the humidity. The reaction mixture was freed from excess hydrazine by evaporation in vacuo. The residue was dissolved in a small amount of water, then the most part of amino acid hydrazides, derived from internal amino acid residues in peptide chains, was removed by precipitation with isovaleraldehyde at pH 7.0.

The clear filtrate containing free amino acids derived from C-terminal residues,

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3) This suspension gave a clear orange-brown solution at pH 3.0.

4) Diethylamine was removed by treating the paper with a current of steam before ninhydrine reaction (*11*).

was treated with dinitrofluorobenzene in the presence of sodium bicarbonate according to the usual method. The DNP-amino acids thus formed were fractionated with ethyl acetate into " C-terminal fraction "[5] (extractable by 2 per cent sodium bicarbonate from ethyl acetate layer) and " tyrosine-fraction "[5] (extractable by bicarbonate from ether layer, but not from ethyl acetate layer).

b) *Identification of DNP-Amino Acids by Paper Chromatography*: The identification of DNP-amino acids was carried out by means of paper chromatography. The elimination of dinitrophenol which interferes with the identification, was performed on paper with a mixture of decalin and acetic acid (1:1) as the developing solvent (descending) (*14*). The band due to dinitrophenol was removed and the slower moving bands including the starting line were cut out, then eluted with 2 per cent sodium bicarbonate. The eluates were extracted with ethyl acetate after acidification and DNP-amino acids in the extracts were identified by chromatography on paper with benzyl alcohol-ethanol (9:1) saturated with an equal volume of phthalate buffer pH 6.0[6] (*15*).

Determination of N-terminal Amino Ccids—Two independent methods were applied for this purpose, PTC-method by Edman (*16*) and DNP-method by Sanger (*2*).

(*I*) *PTC-Method*—

a) *Reactions*: An aqueous solution of the protein component was mixed with the same volume of pyridine (fine uniform suspension). 0.1 ml. phenylisothiocyanate per 50 mg. protein was added and the reaction was carried out under vigorous agitation at 40° for 2 hours keeping the pH at 8–9 by means of sodium hydroxide. After the reaction, pyridine and excess of phenylisothiocyanate were extracted eight times with the same volume of benzene. The faintly yellowish precipitate (phenylthiocarbamyl (PTC)-protein) in the aqueous layer (pH 6–7) was separated by centrifugation, washed with water, ethanol, then with ether (peroxide-free) until the absorption at 270 mμ. was no more observed (extinction coefficient less than 0.02). The PTC-protein was dried in vacuo.

The cyclization of *N*-terminal amino acid residues was carried out by boiling the PTC-protein in *N*-HCl (fine suspension) under reflux. Heating for about 1.5 hours was found to be most suitable for obtaining maximum yield of thiohydantoins. The thiohydantoins formed were quantitatively extracted with ether and the combined extract was washed with water, then used for measuring the ultraviolet absorption (240–280 mμ, max. 268–270 mμ.)

b) *Identification of Thiohydantoins*: The identification of phenylthiohydantoins in the ether extract was carried out by paper chromatography. The ascending technique with two systems of solvents, pyridine-heptane (3:7) (A) and heptane-*n*-butanol-formic acid (80 per cent) (4:2:4) (B), was used for qualitative identification according to Sjöquist (*17*). All the papers were previously treated with starch freed from heavy metals by means of 8-hydroxyquinoline. The color was developed by spraying a

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5) Designated by Akabori and Ohno (*13*).

6) Paper preliminarily treated with the same buffer.

mixture of sodium azide and iodine.

For the semi-quantitative determination of thiohydantoins, a modification of solvent (B), heptane-butanol-formic acid (7:4:6) (B′) was used by descending technique. This solvent gave better separation for glycine and glutamic acid phenyl-thiohydantoins than the other solvents. The intensity and the size of each spot were compared with those of authentic samples of thiohydantoins of known amounts.

c) *Hydrolysis of Thiohydantoins and Identification of Amino Acids Formed*: Hydrolysis of thiohydantoins was carried out by heating with 0.25 *N* barium hydroxide in a sealed tube at 100° for 24 hours. The hydrolyzate was neutralized with sulfuric acid and the amino acids in the supernate was identified by means of paper chromatography with *n*-butanol-acetic acid-water (4:1:5 or 4:1:2) and with phenol-*m*-cresol-borate buffer pH 9.3 (25:25:7) on the buffered paper. The spots were detected by ninhydrine as well as by the specific test for proline with isatin.

(*II*) *DNP-Method*—

The DNP-derivatives of the protein component was prepared by the usual method. The DNP-protein was hydrolyzed by boiling with 6 *N*-HCl for 4 or 8 hours. The DNP-amino acids formed were identified by paper chromatography after extraction with ether. The technique used was essentially the same as the one described above. Two systems of solvents, benzylalcohol-ethanol saturated with phthalate buffer and a phosphate buffer (0.5 M-K_2HPO_4+1.0 M-KH_2PO_4 (1:1)), were used for identification.

RESULTS AND DISCUSSION

C-terminal Amino Acids

(*I*) *Carboxypeptidaes Digestion*—

a) *Identification of C-terminal Amino Acids Liberated by Carboxypeptidase Digestion*: For identifying C-terminal amino acids, a hemoglobin preparation was digested for two hours at 37° by crystalline carboxypeptidase. Amino acids liberated during the enzymatic action were separated from the digest by means of a cationic ion-exchanger, Dowex-50, and submitted to paper chromatography.

Three intense spots and one faint spot were found on the one dimentional chromatogram with butanol-acetic acid. They were identified as leucine, valine, alanine and glycine by their R_f-values as well as by superposing authentic samples of these amino acids on the product by carboxypeptidase digestion. The identity of these amino acids were further confirmed by two-dimensional chromatography with methanol-water-pyridine and tert.-butanol-methylethylketone-water-diethylamine system.

b) *Quantitative Determination of Amino Acids Liberated during the Course*

of Enzyme Action: By comparing a series of chromatograms on which samples obtained at several different times of enzyme action were developed, it was readily seen that three intense spots, corresponding to leucine, valine and alanine, appeared in the early stage of the enzyme action, although the one due to glycine became detectable only after a certain time had elapsed.

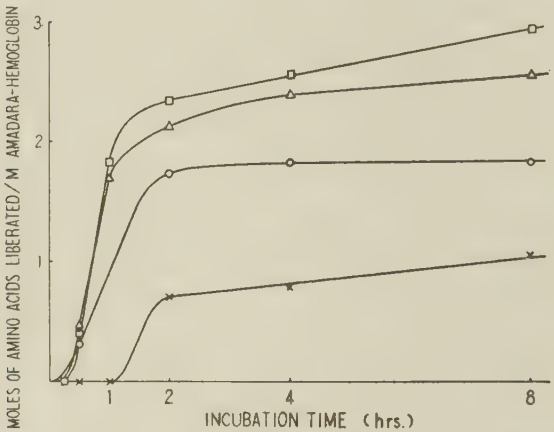


FIG. 1. Rate of liberation of amino acids by the action of carboxypeptidase on *Anadara*-hemoglobin. Enzyme: substrate=1:50, pH 8.1_2, at 37°.

—○—; Leucine. —△—; Valine. —□—; Alanine. —×—; Glycine. The molecular weight of Anadara-hemoglobin=73,000.

An example of the results obtained was shown in Fig. 1, in which the number of each amino acid residues per molecule of hemoglobin (based on the molecular weight of 73,000) was plotted against time. As seen in the figure, the amount of leucine, valine and alanine increased very rapidly in the early stage. But, after this period, only the leucine residue remained on a level of nearly two residues per molecule of hemoglobin, whereas the other two showed rather slow but steady increase during eight hours of digestion. This observation might well be interpreted by assuming that there are two residues each of leucine, valine and alanine present at the C-terminal position of peptide chains and that the slow increase observed in the later stage is due to liberation of valine and alanine residues adjacent to the C-terminal position. On

the contrary to these three amino acids, glycine did not appear at all in the early stage of digestion and became detectable only after two hours. This seemed to indicate that the glycine residue is not present at the C-terminal, but does exist in a position adjacent to it. This point was further examined by Akabori's method, considering the fact that the rate of liberation of C-terminal glycine is the slowest among the

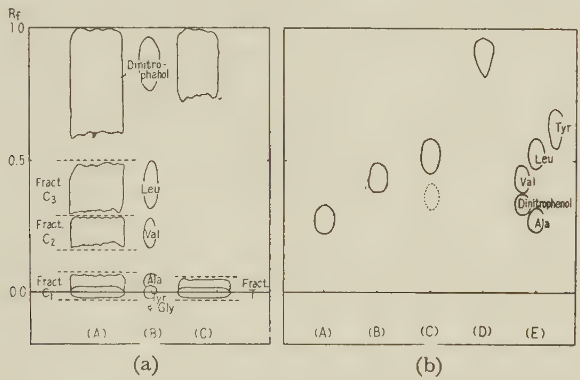


FIG. 2. (a) Paper chromatogram (Tôyôroshi No. 50) of DNP-derivatives formed by hydrazinolysis of *Anadara*-hemoglobin and then dinitrophenylation. (A) "C-terminal fraction"; (B) atuhentic samples of DNP-amino acids; (C) "Tyrosine fraction." Solvent system, decalin-acetic acid (1:1). Time of development was overnight and thus the solvent ran out of the lower end of the paper. The R_f value of each band was calculated, assuming the Rf of dinitrophenol equal to 1.0.

(b) Paper chromatogram (Tôyôroshi No. 50) of each fraction eluted from the above paper chromatogram with 2 per cent sodium bicarbonate for one day. (A) Fraction C_1; (B) Fraction C_2; (C) Fraction C_3; (D) Fraction T; (E) authentic samples of DNP-amino acids. Solvent system, benzyl alcohol-ethanol (9:1).

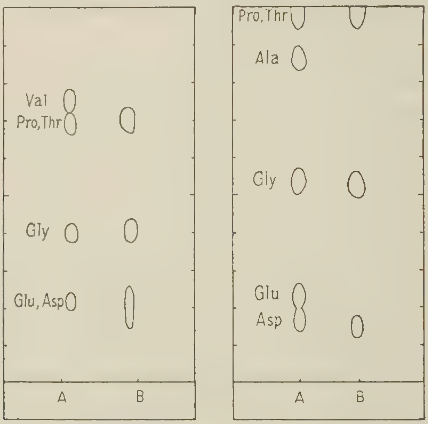
terminal amino acid residues ever known.

(*II*) *Hydrazinolysis*—

This method has an advantage of determining the C-terminal residues more directly than the carboxypeptidase digestion described above.

The amino acid mixture derived from C-terminal residues was dinitrophenylated, then submitted to paper chromatography on a large

paper with decalin-acetic acid. Dinitrophenol formed during the reaction migrated most rapidly and other three yellow bands corresponding approximately to DNP-leucine (Fraction C_3), valine (Fraction C_2) and alanine or glycine (Fraction C_1) were observed in the case of " C-terminal fraction " (Fig. 2a). At the starting line, there remained some material which turned to reddish color in alkaline medium, suggesting the presence of DNP-derivatives of acidic amino acid monohydrazides. Only dinitrophenol and some material of this sort at the



(a) Ascending (b) Ascending

FIG. 3. Paper chromatograms (Tôyôroshi No. 51) of the ether extract (B) of phenylthiohydantoins obtained from the reaction of phenyl-isothiocyanate with *Anadara*-hemoglobin. Solvent system, (a) heptane-pyridine (7:3); (b) heptane-formic acid-*n*-butanol (2:2:1). A, PTH-derivatives of Valine, Proline, Threonine, Glycine, Glutamic acid and Aspartic acid.

starting line (Fraction T) were observed in the case of " Tyrosine fraction." The three bands corresponding to DNP-amino acids (" C-terminal fraction ") and the part around the starting line were extracted separately and the extracts were submitted to paper chromatography with benzyl alcohol-ethanol system (Fig. 2b).

Only DNP-alanine was found in Fraction C_1 and only DNP-valine in Fraction C_2. A faint spot corresponding to dinitrophenol was found with a intense spot due to DNP-leucine in Franction C_3. No spot corresponding to either DNP-tyrosine or other DNP-amino acids was observed in Fraction T except a reddish yellow spot migrating with the solvent

front, probably due to acidic amino acid monohydrazides.

These results, in addition to those obtained by carboxypeptidase digestion, seem to indicate that two residues each of leucine, valine and alanine per molecule do exist at the C-terminal position of *Anadara*-hemoglobin and that glycine, alanine and valine residues might be present adjacent to these terminal residues.

N-terminal Amino Acids

(*I*) *PTC-Method*—

a) *Qualitative Identification*: Both *Anadara*-hemoglobin preparation and its protein component were used for the experiments, but no essential difference was observed between the results obtained on both smaples by qualitative chromatography.

The chromatograms of phenylthiohydantoin (PTH) derivatives of *N*-terminal amino acids were shown in Fig. 3. In several runs it was noted that three main spots were observed with both systems of solvent mixture, A and B, according to Sjöquist. They were identified as PTH-derivatives of proline or threonine, glycine and glutamic acid or glutamine by their R_f-values and by superposing a small amount of authentic samples of these amino acids.[7] The separation of PTH-proline and -threonine could be achieved neither by these solvent systems nor by modifying the proportion of each solvent. Because of the poor reproducibility, it was rather difficult to distinguish PTH-derivatives of glutamic acid, aspartic acid and their amides, but under some favorable conditions the spot in question, which was smaller than the other two spots, was found to be derivative of glutamic acid or glutamine rather than those of aspartic acid. This was confirmed by hydrolyzing the PTH-derivatives with barium hydroxide.

After hydrolysis, two spots were found on the chromatogram with butanol-acetic acid (Fig. 4). The faster migrating spot was identified as proline by its R_f-value and by the specific color reaction with isatin. The slower migrating one occupied a postion between those of glycine and glutamic acid. The spot was eluted and rechromatographed with buffered phenol-m-cresol system. Glycine and a small amount of glutamic acid were identified on the chromatogram.

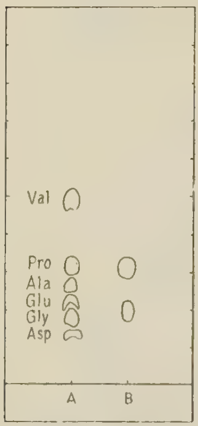
No conclusive result was obtained as to threonine from the above

7) Trace amount of PTH-alanine and -valine was found in some cases, but not investigated further.

experiments, because of its labile nature in alkaline medium used for hydrolysis of PTH-derivatives. This point will be discussed later in the following discussion.

b) Semi-quantitative Determination of PTH-Derivatives: Appreciable difference was observed in the UV-absorption spectra of thiohydantoins depending upon whether hemoglobin or its protein component was used as the starting material.

As seen in Fig. 5, the etherial extract of PTH from hemoglobin



Descending for 20 hours.

FIG. 4. Paper chromatogram (Tôyôroshi No. 50) of amino acids (B) formed by hydrolysis of phenylthiohydantoins from *Anadara*-hemoglobin with 0.25 *N* barium hydroxide, at 100°, for 24 hours in sealed tube. Solvent system, acetic acid-*n*-butanol-water (4:1:2). A, Valine, Proline, Alanine, Glutamic acid, Glycine and Aspartic acid.

FIG. 5. Ultraviolet absorption curve of ether extract (60 ml.) of phenylthiohydantoins obtained from the reaction of phenyl-isothiocyanate with 73 mg. of *Anadara*-hemoglobin or its protein component.

—○—, from hemoglobin —×—, from protein component.

showed its maximum absorption around 260 mμ., whreas the one from the protein component showed a typical absorption curve for PTH

with a maximum at 268–270 mμ. The similar phenomena were observed in the case of horse hemoglobin and its globin component. The number of *N*-terminal residues in horse hemoglobin has been reported as six residues per molecule (valine) (*2*). Assuming that the absorption at the maximum is entirely due to PTH-valine based upon the maximum extinction coefficient regardless of the shift of the peak, a calculation revealed that horse hemoglobin must have nine *N*-terminal residues per molecule when the hemoglobin itself was used as the starting material. This rather improbable high value and the shift of the peak seem to indicate that some unknown compounds with strong absorption in UV region are formed by the reaction of phenylisothiocyanate with the heme component of hemoglobin. By this reason, the protein component freed from heme was used throughout the experiments.

The rate of formation of PTH-derivatives from PTC-protein was first checked by means of UV-absorption (at 270 mμ). The yield of PTH reached to maximum value by heating PTC-protein for 1.5 hours. A similar curve was obtained in the case of PTC-horse-globin. The maximum yield of PTH-derivatives per molecule of protein was 1.4 moles for *Anadara*-protein and 3.8 moles for horse-globin which is known to have 6 valine residues as *N*-terminal. On applying the factor obtained in the latter case to the former case, it was derived that about two or three *N*-terminal residues exist in *Anadara*-hemoglobin. Evidently, this should be regarded as a tentative value, because the nature of *N*-terminal residues might play an important role in such reactions.

The UV-absorption at 320 mμ. was also followed during the above experiment, since PTH-threonine has been known to have a characteristic peak at this position (*18*). No change was observed, thus suggesting the absence of threonine-residues as *N*-terminal groups.

The amount of each hydantoins in the extract was determined on paper chromatogram (solvent B′) by comparing the intensity and the size of each spot with those obtained with various amounts of authentic samples in the same run. Some difficulties arose during the experiments because of the fact that the intensity of the spot due to PTH-proline is much weaker (by a factor of 1/15–1/20) than the one given by the same amount (on molar basis) of PTH-glycine. The purity of PTH-proline was comfirmed by its melting point, *N*-content and paper chromatogram. However, it was finally concluded that 0.7 moles of PTH-proline, 0.6 moles of PTH-glycine and 0.1 moles of PTH-glutamic acid or -glutamine per mole of *Anadara*-hemoglobin are present in the extract. Thus, the existence of proline and glycine residues at the *N*-terminal position of

Anadara-hemoglobin, in an extent of one residue each per molecule at least, seems to be fairly certain, but it is doubtful in the case of glutamic acid or glutamine. This amino acid appeared in all the experiments, but the amount was much less than the other two. This point was further checked by DNP-method described below.

(*II*) *DNP-Method*—

Because of the unstable nature of DNP-proline and -glycine, the purpose of this experiment was restricted to check the possibility whether glutamic acid, described above, or any other amino acids exist at the *N*-terminal position of hemoglobin.

After hydrolyzing DNP-protein with hydrochloric acid, dinitrophenol was eliminated by paper chromatography with decalin-acetic acid (cf. Fig. 2). Only two bands were seen, the faster migrating one corresponding to dinitrophenol and the other one around the starting line corresponding to many DNP-amino acids. The latter band was eluted, then examined by paper chromatography with benzyl alcohol-ethanol and with phosphate buffer as developping solvents. A spot corresponding to DNP-glutamic acid was observed on all of the chromatograms, but its amount never exceeded 0.1 mole per mole of protein. Trace amount of DNP-glycine was seen, but DNP-proline was hardly detectable as expected. No other DNP-amino acid, including alanine and valine of which the likely faint spots appeared in a few cases by PTC-method, was detected on the chromatogram.

The finding of small amounts of glutamic acid by these two independent methods is hardly explainable at present. The splitting of internal peptide bonds adjacent to glutamic acid during the treatment of protein is very unlikely, because these two methods differ greatly in principle. The possibility that glutamic acid might be derived from some contaminant proteins or peptides would be excluded by the facts that the preparations served for use were homogeneous and that no appreciable difference was found in the amount of glutamic acid with the purified preparations before and after crystallization as starting material, unless our preparations are mixtures of very similar proteins, indistinguishable by our physical methods. The latter possibility might be true being a part of hemoglobin molecules deprived of their original *N*-terminal residues, proline and glycine, as it has been shown in the case of some vertebrate hemoglobin (*19*). This point needs further investigation.

SUMMARY

1. Terminal amino acid residues of a crystalline invertebrate hemoglobin from *Anadara inflata* (Reeve) were investigated.

2. Two residues each of leucine, valine and alanine exist at the C-terminal position and glycine, valine and alanine adjacent to it.

3. At least one residue each of proline and glycine seems to be present at the *N*-terminal position. Small amounts of glutamic acid (or glutamine) were always found with these *N*-terminal amino acids, but the significance of this finding is hardly explainable at present.

4. Thus, the protein component of *Anadara*-hemoglobin differs remarkably from those of vertebrate hemoglobins studied so far.

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STUDIES ON THE DENATURATION OF ENZYMES

I. EFFECT OF CONCENTRATION ON THE RATE OF HEAT-INACTIVATION OF ENZYMES

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It was previously reported that sweet potato β-amylase is stabilized against heat by heat-stable component of extracts of leaves of raddish and turnip, and of onion bulb (*1*).

Following the study, it was attempted to characterize such β-amylase stabilizing substances, and to understand the mechanism of the action. For these purposes, however, it is necessary to establish the influences of experimental conditions, such as buffer conditions and enzyme concentration, upon heat-inactivation of the enzyme. No available data for these purposes concerning sweet potato β-amylase have been found in literatures, so far as we know.

As a preliminary experiment, therefore, the influences of some factors upon heat-inactivation have been studied, and some information has been obtained. Especially, it was recognized that there was an quantitative relationship between the rate of heat-inactivation and enzyme concentration, and a provisional experimental equation was introduced.

Sweet potato β-amylase has been crystallized already by Balls *et al.* (*2*), and has been used for experiments by many investigators. Although we also could prepare a small amount of crystals of this enzyme, the amount was too small to be used for our study. Therefore, Balls' "purified paste" which is the step just prior to crystallization, has been used. Activity per mg. nitrogen of this preparation was about 50 per cent of Balls' crystal.

Preparation of crystalline enzyme is now in progress in our laboratory. Experiments will be repeated, when sufficient quantity of crystal is available, to test whether any difference due to the purity is present.

EXPERIMENTAL

Amylase—Aqueous solution of Balls' "purified paste" of sweet potato β-amylase was dialyzed against deionized water for 4 to 5 hours, and used in all experiments.

Heating Methods—The enzyme solution in acetate buffer was heated in the bath of $63 \pm 0.1°$ in the following two manners.

1. The enzyme solution in a test-tube of 30 mm. diameter, was heated rapidly in the constant temperature bath. After the temperature of solution reached to 63°, an aliquot of the solution was withdrawn at regular intervals of time, cooled immediately in ice water to stop the inactivation, and then the remaining activity was measured.

2. Several test-tubes of 15 mm. diameter, each containing 2 ml. of enzyme solution, were immersed in the bath simultaneously, and were heated. Each of test-tubes was withdrawn at the time as indicated above. The rest of the procedures taken were the same.

The time until the temperature of solution reached to 63°, was less than 1 minute even at 10° of room temperature.

Activity Measurements—0.5 ml. of suitably diluted enzyme solution, 0.1 ml. of $M/2$ acetate buffer (pH 5.4), and 1.0 ml. of 5 per cent soluble starch solution were mixed in total volume 2.0 ml., which was incubated at 40° for 5 minutes. Then the increase in reducing power was measured by means of micro-Bertrand procedure, and was represented as mg. of glucose. The velocity constant was calculated by the equation: $k = 2.3/t \log(a_i/a_f)$, where, a_i and a_f are activities of enzyme at zero time, and at t minutes later, respectively, and k is the first-order rate constant.

RESULTS AND DISCUSSION

1. *Heat-Inactivation Process of β-Amylase*—Sweet potato β-amylase in acetate buffers of varying pH, was heated at 63°, and logarithms of residual activities were plotted against time. As illustrated in Fig. 1, straight lines were obtained in the range of these experimental conditions, and it appeared that heat-inactivation of β-amylase follows first-order kinetics with respect to time. Therefore, the inactivation rates were represented as first-order rate constants in the following experiments.

2. *Influences of pH and Buffer Concentration upon Heat-Inactivation*—β-Amylase was heated at 63° in acetate buffers of different pH, the residual activities were measured at pH 5.4, and then rate constants of inactivation were calculated. The pH of buffers were measured with glass

electrode pH-meter, Mitamura & Co., Ltd. The result of this experiment is shown in Fig. 2. Amylase appears to be stable between pH 5.0 and 5.8, and the most stable pH is about 5.4. The stability of amylase markedly decreases as pH shifts to more acid or more alkaline side of this pH.

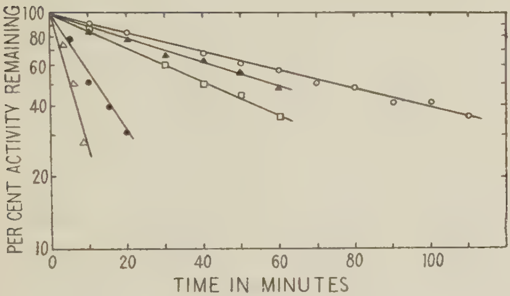

FIG. 1. Heat-inactivation curves of β-amylase in some conditions at 63°.

Conditions as follows:

○, 0.02 *M* acetate buffer, pH 5.4
▲, 0.05 *M* ,, ,, , ,, 5.6
□, 0.05 *M* ,, ,, , ,, 5.0
●, 0.05 *M* ,, ,, , ,, 4.5
△, 0.01 *M* ,, ,, , ,, 4.0

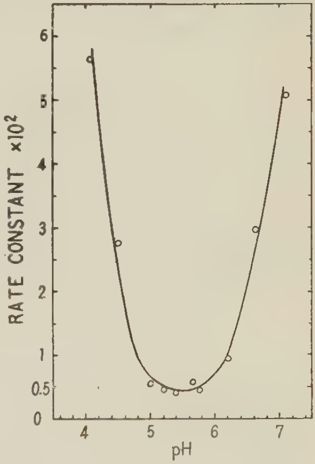

FIG. 2. Influence of pH upon heat-inactivation.

Enzyme in 0.01 *M* acetate buffer was heated at 63° at intervals, and calculated constants were averaged. Buffer in activity measurement: 0.125 *M* acetate buffer (pH 5.4).

The optimum pH of crystalline sweet potato β-amylase is reported to be between 4 and 5 (*2*), and its isoelectric point lies between 4.74 and 4.79 (*3*). The most stable pH obtained in our experiment, therefore, is in a slightly more alkaline region than those optimum pH values. And at pH 4, where enzyme is in optimum active region, it is extremely unstable to heat. Further, in contrast with the broader plateau of activity-pH curve of amylase, the range of its heat-stability is much more narrow. However, since enzyme preparation used in this study is not sufficiently pure, the possible influences of impurities upon these results may have to be taken into consideration. Experiments with buffers

other than acetate, have not so far been carried out.

As to the influence of buffer concentration, amylase is more stable in lower concentration in the region between 0.01 and 0.1 *M* buffer concentration, as shown in Table I. However, since the ionic strength of buffer was not controlled in this experiment, it can not be concluded whether the influence of buffer concentration is due to undissociated acetate molecules or to acetate ions.

TABLE I

Influence of Buffer Concentration upon Heat-Inactivation

	Buffer concentration					
	0.01	0.02	0.04	0.06	0.08	0.1 *M/l.*
$k \times 10^3$	4.18	4.42	4.70	5.62	6.61	8.50

Rate constants are averaged values, cf. Fig. 2.; pH 5.4, 63°.

3. *Effect of Enzyme Concentration*—The heat-inactivation of enzymes are often affected by concentration of enzymes themselves. As to amylase, M. L. Caldwell *et al.* (*4*) have reported that crystalline pancreatic amylase is more stable in more concentrated aqueous solution.

Sweet potato β-amylase is also more stable at 63° and 64°, as its initial concentration is increased in the region of concentrations of these experiments, as illustrated in Table II.

The kinetics of heat-denaturation of proteins, including enzymes, have been studied by many investigators, as reviewed by F. W. Putnam (*5*). It has been reported that many of them denature following first-order kinetics. Sweet potato β-amylase used in our study was also inactivated following first-order kinetics with respect to time, in our experimental conditions. This suggests the inactivation reaction is of intramolecular nature.

Proteins are generally more stable in concentrated than in dilute solution. That the rate of heat-inactivation of sweet potato β-amylase decreases as enzyme concentration increases, coincides with the case of pancreatic amylase, and also with ordinary proteins.

By plotting logarithms of rate constants of inactivation shown in Table II (k), against logarithms of the concentration of enzyme solution yet to be treated (C_i), straight lines were obtained as illustrated in

TABLE II

Influence of Concentration of Amylase upon its Heat-Inactivation

Relative enzyme concentration	Rate constant ($\times 10^3$)	
	at 63°	at 64°
6	18.9	30.2
8	13.9	24.0
10*	6.86	16.5
15	4.10	12.0
20	2.53	9.2
30	0.805	

* 0.002 mg. N/ml.

Rate constants are averaged values, cf. Fig. 2.; 0.05 *M* acetate buffer, pH 5.4.

Fig. 3. The following apparent equation, therefore, will be formulated concerning k and C_i:

$$k=\alpha C_i^{-\beta}$$

where, α and β are constants in the inactivation condition. And β appeares to be a function of temperature, since straight lines of 63° and 64° have different slopes.

Since the enzyme used in this study, however, was not crystalline pure, the impurity might have affected on the experimental results. A similar experiment was, therefore, carried out with crystalline Taka-amylase solution, which was supplied by Akabori Laboratory, Faculty of Science, Osaka University, and the rate constants were plotted in the like manner. The result is illustrated in Fig. 4. Here again, straight line was obtained, and an equation of the same form as the partially purified sweet potato β-amylase was introduced.

On the other hand, Caldwell *et al.* (*4*) described nothing about the rate constants, and their experimental data are not appropriate for the calculation of the correct constants, the activity of an unheated enzyme of only a single concentration was taken as control. However, when relative rate constants, calculated from their data, were plotted in our manner, straight line was similarly obtained.

Haurowitz *et al.* (*6*) have reported that, the heat-denaturation of ovalbumin follows first-order kinetics with respect to protein con-

centration, but higher order with respect to time. And they have discussed the mechanism of this dualism.

In the present study, heat-inactivation of β-amylase follows first-order kinetics with respect to time (Fig. 1). But the rate constant with respect to time calculated according to first-order equation, is a function of the concentration of intact enzyme solution. These results may

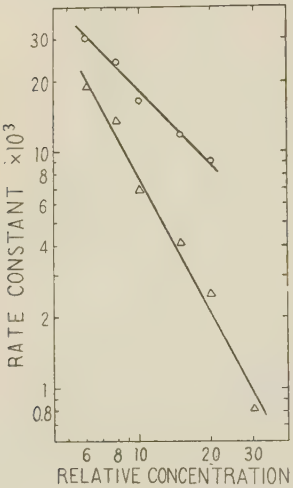

Fig. 3. Log k-log C_i curves of heat-inactivation.

△ represents constants obtained at 63°.

○ represents constants obtained at 64°.

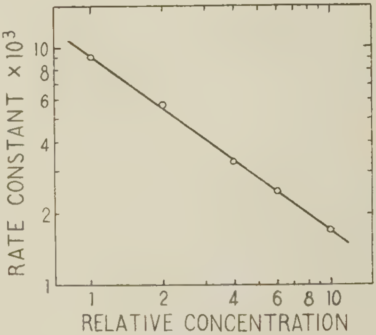

Fig. 4. Log k-log C_i curve of crystalline Taka-amylase.

Relative concentration "10", about 1/50 per cent crystal; M/20 ammonium acetate (pH 6.6); 51±0.1°.

Activity measurement: heated and diluted enzyme 0.5 ml., 0.01 M Ca acetate 0.1 ml., 0.5 M acetate buffer (pH 5.4) 0.4 ml., 5 per cent soluble starch 1.0 ml., total volume 2.0 ml.; 40°, 5 minutes.

suggest that, the stoichiometrical concentration of intact enzyme solution means not merely the amount of enzyme protein molecules, but some function which affects the physical property of protein, and that, some states of enzyme protein molecule vary with its stoichiometrical concentration, and such molecule that predominates in more concentrated solution, will be more stable to heat than the other.

SUMMARY

The influences of several factors upon heat-inactivation of sweet potato β-amylase, were studied:

1. The inactivation appeared to follow first-order kinetics with respect to time.

2. In the experimental range of pH 4 to 7, β-amylase in acetate buffer was stable in the range of about pH 5.0 to 5.8, and the most stable pH was about 5.4.

In the range of 0.01 to 0.1 *M* of acetate buffer, the less concentrated the buffer, the more stable was β-amylase.

3. The more concentrated β-amylase solution, the more stable was the enzyme. And the following apparent equation may be formulated concerning first-order inactivation rate constant with respect to time (k), and the stoichiometrical concentration of intact enzyme solution (C_i):

$$k = \alpha C_i^{-\beta}$$

where, α and β are constants. This may suggest that the physical property of enzyme protein molecule is a function of the concentration of intact enzyme solution.

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STUDIES ON THE INTERACTION OF GELATIN WITH LEAD ION*

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Studies on the interactions of proteins with metallic ions in solution are very valuable not only for understanding the role of metals in the physiological phenomena but also for analysing the surface structure of protein molecules.

For this purpose, numerous experimental methods have been devised, among which the method of dialysis equilibrium used by Klotz and other investigators (*1*) seems to be most promising. It was pointed out, on the other hand, by Tanford (*2*), Breyer (*3*), and others (*4*) that the polarographic method is also very useful for the investigation of the interaction of proteins with metallic ions or some dyestuffs.

Although it has become apparent that the active sites of proteins for the metal-protein interaction are mainly the charged groups of side chains in a protein molecule, the role of the higher order structure of protein molecules in this phenomenon is also an important problem to be solved. Some light will be thrown to this problem by studying the metal-protein interactions using both the globular and fibrous proteins and by comparing their experimental results. We have recently studied by the polarographic method the interaction of gelatin with Pb^{2+} ion as an example of the fibrous protein and metallic ion systems, the results of which will be discussed in the following and compared with the results obtained by other investigators with the system of globular protein and cation or anion (*5*).

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EXPERIMENTS

Reagents

Gelatin—The granular gelatin, which was made from the bovine leather by Fuji Factory of the Japan Leather Co., was used, the ash content of which was less than 1.4 per cent as the sulfate salt. The solution of gelatin was made by dissolving a known quantity of gelatin into a certain amount of distilled water with continuous stirring in a gently boiling water-bath. The concentration of gelatin solution was determined by the following method: Evaporate a known quantity of the solution to dryness in a boiling water-bath; dry the sample in an electric oven at 120° for 30 minutes; weigh the sample, and repeat the drying and weighing until the constant weight of the sample is obtained.

The pH of one percent gelatin solution (measured by a glass electrode pH-meter) was 6.2_0. The solution was further purified by flowing it through a glass column, which contained the ion-exchange resins, IR-120 and IRA-410 of Rohm and Haas Co.; the outside of the column was covered with a jacket containing hot water to prevent the gelation of the gelatin solution. By this procedure the pH of the solution changed to 5.3_0. The interaction of gelatin with Pb^{2+} ion was checked by using both the original and the purified gelatin solutions, the results of which did not show any remarkable difference in these two cases. Therefore, the original gelatin solution was used throughout the following experiments.

Polyoxyethylene Lauryl Alcohol Ether (*LEO*)—LEO of the highest purity, prepared by Nezu Chemical Laboratory, Tokyo, was used as the maximum suppressor in the polarographic determination of the free Pb^{2+} ion.

All other chemicals were reagent grade and used without further purification.

Determination of the Adsorption Isotherms of the Gelatin-Pb^{2+} ion-Indifferent Salt Systems—The adsorption isotherms of the reacting systems, which contained the large quantity of indifferent salts in comparison with the concentration of Pb^{2+} ion, were obtained by measuring the concentration of the free Pb^{2+} ion in the system.

This was done with the polarographic method by comparing the diffusion current due to the free Pb^{2+} ion in a certain reacting system with the standard curve showing the relation between the diffusion current and the concentration of the Pb^{2+} ion, which was obtained with the solutions consisting of the various concentrations of Pb^{2+} ion, the same concentration of the indifferent salt as the reacting system and 0.0001 M LEO as the maximum suppressor. This method is based upon the assumption that the diffusion current of the Pb^{2+} ion is not affected by the presence of the relatively high concentration of gelatin. Although the presence of gelatin changes the viscosity of the solution, this assumption seems to be plausible, because it has been known that the diffusion coefficient of such a small ion as Pb^{2+} ion is not influenced by the change of viscosity of the solution due to the presence of macro-molecules such as gelatin (*6*). It is also assumed that the diffusion current due to the reduction of the gelatin-Pb^{2+} ion complex, if such a complex is reduced, is negligibly small in comparison with that of the free Pb^{2+} ion.

Under the present experimental conditions, the equilibrium of the interaction was found to be reached within about 30 seconds from the initiation of the reaction. Therefore, all measurements were carried out after standing the reacting system for *ca.* 1 hour from the initiation of the reaction.

The apparatus used was a pen-recording YOL-I polarograph (Yokogawa Electric Works Co.); Ag/AgCl electrode, which was connected to the cell solution with a KCl-agar bridge, was used as the anode. The dissolved oxygen in the cell solution was expelled by gently bubbling nitrogen through the solution.

The Measurement of the Viscosity—The viscosity of the reacting system was measured by using a U-type Ostwald viscometer, through which 10.0 ml. of distilled water flowed in 96.3 seconds at 25.0°.

RESULTS

Test of the Reversibility of the Gelatin-Pb^{2+} Ion Interaction—After the equilibrium of the interaction had been reached with the system, 0.177 per cent gelatin-Pb^{2+} ion-0.1 *M* NH_4Cl, at 25.0°, the concentration of the free Pb^{2+} ion in the system, was determined. This solution was dialysed through a cellophane membrane against the flowing distilled water for a long period, by which the concentration of the free Pb^{2+} ion in the system was greatly reduced. A known quantity of Pb^{2+} ion was then added to the dialysed system and the concentration of the free Pb^{2+} ion in the system at the equilibrium was found to be lower than that corresponding to the added Pb^{2+} ion. This result means that the initially combined Pb^{2+} ion was largely dissociated and removed from the system by the dialysing process and the gelatin again interacted with the newly added Pb^{2+} ion. The reversibility of the interaction was thus qualitatively confirmed.

Adsorption Isotherm with Various Gelatin Concentrations—Adsorption isotherm of the system, gelatin-Pb^{2+} ion-0.2 *M* KNO_3, was determined with the concentration range of the free Pb^{2+} ion from 0.2 to 8 m*M*. Four concentrations of gelatin, 0.404 per cent, 0.195 per cent, 0.078 per cent, 0.039 per cent, were used. An example of the adsorption isotherms is shown in Fig. 1 (Curve A), where (A) represents the concentration of free Pb^{2+} ion in the system and r the degree of combination in moles of combined Pb^{2+} ion per one gram of gelatin. At the higher concentration of gelatin more than 0.404 per cent the gelation of the solution occurred at 25.0°, and at the lower concentration of gelatin below 0.039 per cent the amount of the combined Pb^{2+} ion was so small that the accurate measurement could not be carried out.

The Effect of the Indifferent Salts—The effect of the indifferent salts

on the gelatin-Pb^{2+} ion interaction was studied with the systems which consisted of 0.2 per cent gelatin, Pb^{2+} ion, and the four kinds of indifferent salts (KNO_3, KCl, NH_4Cl, and $CaCl_2$). Similar curves to the curve A in Fig. 1 were obtained as the adsorption isotherm in every cases. Although the gelatin-Pb^{2+} ion system without any indifferent salt and the system containing 0.1 *M* NH_4Cl were transparent over the whole concentration range of Pb^{2+} ion, the systems, which contained 0.2 *M* KNO_3, 0.2 *M* KCl or 0.1/3 *M* $CaCl_2$, respectively, became turbid at a certain concentration of Pb^{2+} ion and produced precipitates at its higher concentrations.

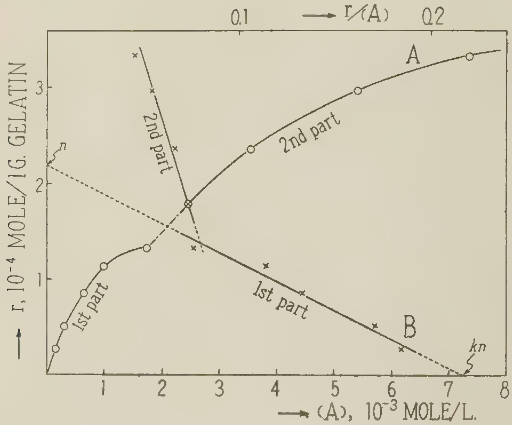

Fig. 1. Adsorption isotherm of the system 0.195 per cent gelatin-Pb^{2+} ion-0.2 *M* KNO_3 at 25.0° and its analysis: A (—○—), degree of combination *r vs.* concentration of free Pb^{2+} ion (A); B (—×—), relation between *r* and *r*/(A).

The Effect of the Ionic Strength—The determination of the adsorption isotherm was carried out at 25.0° with systems containing 0.2 per cent gelatin, Pb^{2+} ion, and the various concentrations of NH_4Cl (0.1, 0.2, 0.3, and 0.5 *M*), in order to study the effect of the ionic strength, μ, of the solution on the gelatin-Pb^{2+} ion interaction. With the systems of μ=0.1 and 0.2, the solution was transparent over the whole concentration range of the Pb^{2+} ion. The solutions of μ=0.3 and 0.5, however, produced precipitates at the concentration of Pb^{2+} ion higher than 6 m*M* and 4 m*M*, respectively. There was found little change in the pH of the solution with the ionic strength.

The Effect of pH—As a discontinuity was found in the adsorption isotherm of each system as shown in Fig. 1 (Curve A), the effect of pH of the solution on the gelatin-Pb^{2+} ion interaction was investigated with two systems containing 0.191 per cent gelatin, 0.1 M NH_4Cl, 1×10^{-4} M and 3×10^{-3} M Pb^{2+} ion, which corresponded to the left and right sides of the discontinuous point in the adsorption isotherm, respectively. The pH of the solution was adjusted by adding HCl to the system. The result is shown in Fig. 2, where the ordinate, $(r/(A))_{pH}/(r/(A))_{max}$, means the ratio of $r/(A)$ value at a certain pH to the $r/(A)$ value corresponding to the maximum pH examined.

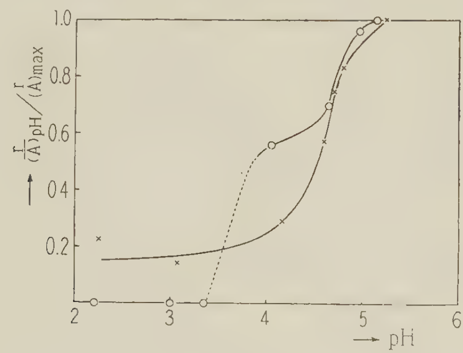

FIG. 2. Effect of pH on the gelatin-Pb^{2+} ion interaction with the system 0.191 per cent gelatin-Pb^{2+} ion-0.1 M NH_4Cl at 25.0°: —×—, concentration of Pb^{2+} ion 1×10^{-4} M; —○—, concentration of Pb^{2+} ion 3×10^{-3} M.

The Effect of Temperature—The effect of temperature was studied at 15.0°, 20.0°, 25.0° and 30.0°, by using the system containing the 0.2 per cent gelatin, 0.1 M NH_4Cl, and various concentrations of Pb^{2+} ion. The shape of each adsorption isotherm obtained was similar to the curve A in Fig. 1. At the temperatures of 15.0° and 20.0°, the reacting solution was turbid in the higher concentration range of Pb^{2+} ion, but at 25.0° the solution was transparent over the whole concentration range of Pb^{2+} ion. A little amount of precipitates was found in the higher concentration range of Pb^{2+} ion at the temperature of 30.0°.

The Measurement of Viscosity—As described above, the solution of the systems containing 0.2 M KNO_3 or 0.2 M KCl as the indifferent salt become turbid in the higher concentration range than 2×10^{-3} M of

Pb^{2+} ion. This shows that a certain change of the dispersion state in the solution occurs at this concentration of Pb^{2+} ion. In order to make this point clearer, the viscosity of the solution, which consisted of 0.194 per cent gelatin, 0.1 *M* NH_4Cl, and the various concentrations of Pb^{2+} ion, was measured at 25.0°. The systems consisting of 0.1 *M* NH_4Cl and Pb^{2+} ion were taken as the solvent for the calculation of the specific viscosity, η_{sp}, of the reacting solution. The relation between η_{sp} of the solution and the concentration of free Pb^{2+} ion, (A), is shown in Fig. 3 (Curve B). The system, which contained 0.1 *M* NH_4Cl as the indifferent salt, was employed in this experiment, because the so-

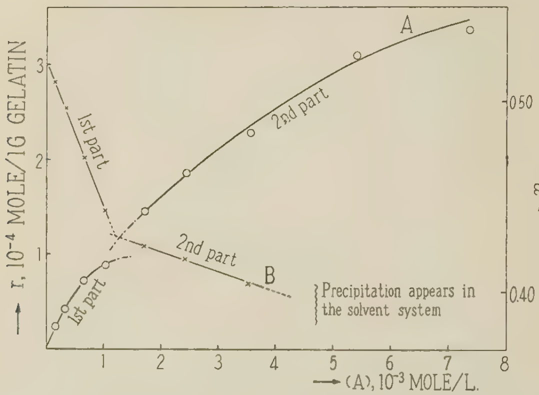

FIG. 3. Adsorption isotherm and specific viscosity of the system 0.194 per cent gelatin-Pb^{2+} ion-0.1 *M* NH_4Cl at 25.0°: A (—○—), degree of combination *r* *vs.* concentration of free Pb^{2+} ion (A); B (—×—), specific viscosity η_{sp} *vs.* (A).

lution of this system was transparent up to the concentration of Pb^{2+} ion, 6×10^{-3} *M*.

DISCUSSION

From the experimental results mentioned in the previous section, it was shown that there was an apparent discontinuity in the adsorption isotherm of each gelatin-Pb^{2+} ion-indifferent salt system (Curve A in Figs. 1 and 3). Such discontinuities have already been reported by Suzutani (*7*) in the concentration range of Pb^{2+} ion less than 2×10^{-3} *M*. He explained this phenomenon by assuming that the various kinds

of sites of different adsorbability gradually come into play as the concentration of Pb^{2+} ion in the system increases.

We found that the relation between η_{sp} of the solutions of gelatin-Pb^{2+} ion-0.1 *M* NH_4Cl system and the concentration of free Pb^{2+} ion was composed of the two straight lines of different inclination (Curve B of Fig. 3). The concentration of free Pb^{2+} ion, at which these two lines intersect, coincides with the concentration of free Pb^{2+} ion where the discontinuity appears in the adsorption isotherm and the solution becomes turbid in certain systems. Therefore, the discontinuity on the viscosity-concentration curve and on the adsorption isotherm should be ascribed to the same cause, which will be explained as follows. With the increase of the amount of Pb^{2+} ion adsorbed, the free electric charge on gelatin molecules will decrease. This effect will bring about the contraction of coiled molecules of gelatin and hence lower the specific viscosity of the solution. After a certain amount of Pb^{2+} ion has combined with gelatin molecules, the dispersion state of the gelatin-Pb^{2+} ion complexes in the solution will become unstable by the decrease of intermolecular electrostatic repulsion and hydration. The further increase of the concentration of Pb^{2+} ion may result in the association or the coagulation of gelatin-Pb^{2+} ion complexes, probably partly owing to the bridging between the complex molecules by Pb^{2+} ion. In this stage some kinds of physical adsorption of Pb^{2+} ion on the associated complexes may occur.* Such a change in the dispersion state of gelatin molecules causes a discontinuity in the viscosity curve and the adsorption isotherm.

The effect of pH of the solution to the degree of interaction (Fig. 2) is due to the change of the degree of dissociation of the various ionic groups in the gelatin molecule. Fig. 2 shows that the degree of interaction greatly increases within the pH range between 4 and 5.2, while it is quite small in the solutions of pH lower than 4. This means that the imidazol and/or carboxyl groups, which are dissociated in the pH range higher than 4, are responsible for the specific interaction of gelatin with Pb^{2+} ion. It was reported by Tanford (*2*), that Pb^{2+} ion interacts with the dissociated imidazol groups in the case of bovine serum albumin while Gurd and Murray (*9*) gave the conclusion that Pb^{2+} ion mainly combines with the dissociated carboxyl groups in the case of horse serum albumin. Klotz and Curne (*10*) also described that many cations

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* Here it may be valuable to cite a report (8) that the combining amount of phosphate ion to serum albumin increases when its solution changes from sol to gel.

interact with the dissociated imidazol groups of the serum albumin molecule. From our present experimental results, it is impossible to explain the problem, which of these two groups is responsible for the gelatin-Pb^{2+} ion interaction. It must be noted, however, that the effect of pH on the gelatin-Pb^{2+} ion interaction is the same as that obtained with the globular proteins.

As the interaction of gelatin with Pb^{2+} ion was found to be reversible at least qualitatively, it is possible to apply the thermodynamic analysis to the experimental results. Let us consider the interaction of gelatin with Pb^{2+} ion is represented by the following multiple equilibrium:

$$\left.\begin{array}{l} \mathrm{P} + \mathrm{A} \rightleftharpoons \mathrm{PA} \\ \mathrm{PA} + \mathrm{A} \rightleftharpoons \mathrm{PA_2} \\ \cdot\ \cdot\ \cdot\ \cdot\ \cdot\ \cdot \\ \mathrm{PA}_{n-1} + \mathrm{A} \rightleftharpoons \mathrm{PA}_n \end{array}\right\} \tag{1}$$

where P means the gelatin molecule, A Pb^{2+} ion, and n the maximum number of binding sites to which Pb^{2+} ion can be adsorbed. The equilibrium constants for each process in Eq (1) are:

$$\left.\begin{array}{l} k_1 = \dfrac{(\mathrm{PA})}{(\mathrm{P})\ (\mathrm{A})} \\ k_2 = \dfrac{(\mathrm{PA_2})}{(\mathrm{PA})\ (\mathrm{A})} \\ \cdot\ \cdot\ \cdot\ \cdot\ \cdot\ \cdot\ \cdot\ \cdot \\ k_n = \dfrac{(\mathrm{PA}_n)}{(\mathrm{PA}_{n-1})\ (\mathrm{A})} \end{array}\right\} \tag{2}$$

in which (P), (A),...represent the concentrations of the corresponding substances. By using this equation, the degree of combination, r, which is defined by the relation

$$r = \frac{(\mathrm{PA}) + 2\,(\mathrm{PA_2}) + \ldots\ldots + n\,(\mathrm{PA}_n)}{(\mathrm{P}) + (\mathrm{PA}) + (\mathrm{PA_2}) + \ldots\ldots + (\mathrm{PA}_n)} \tag{3}$$

can be written as

$$r = \frac{k_1(\mathrm{A}) + 2k_1k_2\,(\mathrm{A})^2 + \ldots\ldots + n\,(k_1k_2\ldots k_n)\,(\mathrm{A})^n}{1 + k_1(\mathrm{A}) + k_1k_2\,(\mathrm{A})^2 + \ldots\ldots + (k_1k_2\ldots k^n)\,(\mathrm{A})^n} \tag{4}$$

Unless there is any electric interaction between the sites or between the bound A and the free A, the equilibrium constant, k_i, can be represented by

$$k_i = \frac{n-i+1}{i} k \ , \quad i=1, 2, \ldots\ldots n \tag{5}$$

where k is the so-called intrinsic equilibrium constant. From these equations we can obrain the relation between the degree of combination, r, and the concentration of the free ion, (A) (*11*):

$$\frac{r}{(\mathrm{A})} = kn - kr \tag{6}$$

An example of the analysis of the adsorption isotherm by using Eq. (6) is shown in Fig. 1 (Curve B). In every cases, the relation between $r/(\mathrm{A})$ and r is consisted of two straight lines; the first part corresponds to the lower concentration and the second part to the higher concentration of Pb^{2+} ion. Owing to the relatively large deviation of the data from the straight line in the second part, where the gelatin-Pb^{2+} ion complexes are supposed to coagulate or associate, it is difficult to apply the strict thermodynamic analysis to the second part.

The values of the maximum number of the site, n, and the intrinsic equilibrium constant, k, can be calculated by applying the least square method to the alternative form of Eq. (6):

$$\frac{1}{k}\frac{1}{(\mathrm{A})} = n\frac{1}{r} - 1 \tag{7}$$

Tables I–IV show the values of n and k thus obtained under the various experimental conditions.

TABLE I

The Effect of the Concentration of Gelatin at 25.0°

(Indifferent salt: 0.2 M KNO_3)

Concn. of gelatin, (%)	n, (mole/g. gelatin)	k
0.039	1.5×10^{-4}	3.9×10^{3}
0.078	1.8 〃	1.2 〃
0.195	2.1 〃	1.0 〃
0.404	2.1 〃	0.9 〃

TABLE II

The Effect of the Indifferent Salt at 25.0°

Indifferent salt	n, (mole/g. gelatin)	k
0.1 M NH_4Cl	2.2×10^{-4}	0.7×10^3
0.2 M KNO_3	2.1 ,,	1.0 ,,
0.2 M KCl	1.7 ,,	0.6 ,,
0.1/3 M $CaCl_2$	2.0 ,,	0.6 ,,

TABLE III

The Effect of the Ionic Strength at 25.0°
(Indifferent salt: NH_4Cl)

Ionic strength	n, (mole/g. gelatin)	k
0.1	2.2×10^{-4}	0.7×10^3
0.2	2.5 ,,	0.5 ,,
0.3	1.4 ,,	0.4 ,,

TABLE IV

The Effect of Temperature
(Indifferent salt: 0.1 M NH_4Cl)

Temperature, °C	n, (mole/g. gelatin)	k
15.0	2.2×10^{-4}	0.8×10^4
20.0	2.2 ,,	0.7 ,,
25.0	2.2 ,,	0.7 ,,
30.0	2.2 ,,	0.6 ,,

It is pointed out that the value of n remains nearly constant, being independent of the concentration of gelatin, kind of the indifferent salt, or the temperature. The change of the ionic strength, however, seems to give some effect on the value of n, *i.e.*, it decreases from *ca.* 2×10^{-4} to *ca.* 1×10^{-4} mole/(1 g. gelatin) with the increase of the ionic strength, μ, from 0.2 to 0.3. With the solution of $\mu=0.5$, the result is very inaccurate because of the large dispersion of the experimental data, but the value of n seems to decrease further. As the pH of the solutions corresponding to $\mu=0.2$ and $\mu=0.3$ is nearly the same, this result will

be due to the difference in the electric field of the solution.

The fact that n is kept nearly constant regardless to the change of the concentration of gelatin is quite remarkable if it is compared with the experimental results of Breyer *et al.* on the interaction of bovine plasma albumin with methyl orange (*3*). They reported that the number of the site, to which methyl orange was combined, increased greatly with the dilution of bovine plasma albumin in its concentration range lower than 0.1 per cent. They have interpreted this interesting result by assuming the appearance of new active sites due to the unfolding and splitting of the bovine plasma albumin molecule accompanying the dilution. However it would be desirable that this assumption is confirmed by employing other proteins, particularly fibrous protein. The molecule of gelatin in our experiments is quite different from that of bovine plasma albumin in that the shape of the former is fibrous while that of the latter is globular, and that the gelatin molecule would be completely denatured during its production process. Therefore, any unfolding or splitting of the molecule with the dilution could not occur in the case of gelatin. Our experimental result, although indirect, could be an experimental support to Breyers' assumption on the change of n of bovine plasma albumin with the dilution.

As described previously, the value of n corresponding to the first part of the adsorption isotherm is independent of the temperature in the range of 15.0° and 30.0°. The intrinsic equilibrium constant, k, however, decreases slightly with the increasing temperature (Table IV). Although the range of temperature studied is very narrow and the data are not so accurate, the approximate values of changes of free energy, heat content and entropy, ΔF, ΔH and ΔS, of the gelatin-Pb^{2+} ion interaction (first part) are obtained to be -3.9 kcal./mole, -2.6 kcal./mole and 4.2 cal. deg.$^{-1}$/mole, respectively*. The present value of ΔF is approximately equal to that in the case of the interaction (*10*), (*12*) of globular protein and Cu^{2+} or Co^{3+} ion. The change of heat content, ΔH, however, is of small negative value and the entropy change, ΔS, is very much lower than that in the case of the globular protein-cation or anion system whose entropy change is around 20 cal. deg.$^{-1}$/mole. These results may be interpreted by the difference in the molecular configuration of globular protein (*e.g* bovine serum protein) and fibrous protein (gelatin). The possible significance of positive entropy changes has been interpreted by the release of hydrated water molecules

* These values were calculated from the formula, $\Delta F = -RT \ln k$.

accompanying the transfer of metal ions from solvent to protein (*10*). However there exists also a possibility that a portion of the entropy increase associated with protein-cation interaction arises from structural alteration of the protein molecule (*13*). The fact that the value of ΔS of the gelatin-Pb^{2+} ion interaction is smaller than that of globular protein-metallic ion interaction suggests that, in the latter case, there occurs much more structural modification of protein molecule through its binding with metallic ions.

SUMMARY

The interaction of gelatin with Pb^{2+} ion was investigated under various conditions by means of the polarographic method. The mechanism of this interaction was analysed by applying the thermodynamic method to the adsorption isotherm, together with the experimental results of the viscosity measurement of the reacting systems. The results obtained are summarized as follows:

1. The interaction is divided into two parts, the first of which corresponds to the lower concentration range and the second of which to the higher concentration range of Pb^{2+} ion.

2. It is concluded that Pb^{2+} ion interacts specifically with the dissociated imidazol and/or carboxyl groups of the gelatin molecule. In the second part, some kinds of physical adsorption of Pb^{2+} ion to the gelatin-Pb^{2+} ion complexes are supposed to occur.

3. In the first part of specific interaction, the value of ΔF is about -3.9 kcal./mole, and the effect of pH is large, which is in agreement with the results of the globular protein-metallic ion interaction. The value of ΔS, however, is much smaller than that obtained with the globular proteins, and that of ΔH is of small negative value.

4. The maximum number of the site available to the specific gelatin-Pb^{2+} ion interaction is kept nearly constant under the various conditions. Especially it is independent of the dilution of gelatin, while its increase with the dilution has been reported by Breyer *et al.* in the case of the bovine plasma albumin-methyl orange interaction. This is supposed to be an important difference between the fibrous (and completely denatured) protein and the globular (and native) protein.

After the completion of this study, one of the present authors (R. T.) had an opportunity of making the valuable discussion with Dr. B. Breyer of the University of Sydney, to whom the present authors express their thanks for his interest in this study.

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ENZYMATIC PREPARATION OF OPTICALLY ACTIVE ESSENTIAL AMINO ACIDS

II. THE PREPARATION OF L-TRYPTOPHAN

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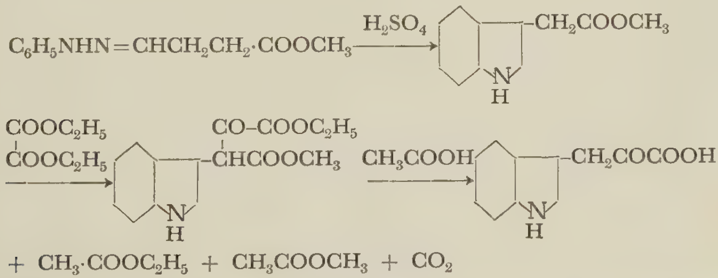
In the previous paper of this series (*1*), a method for the preparation of L-phenylalanine by enzymatic transamination was described. In the present work, the same method was applied for the preparation of L-tryptophan, which has been not easily accessible from natural proteins or by any synthetic methods.

The present method involves two steps, namely, preparation of indolepyruvic acid and the conversion of this to L-tryptophan by transaminase.

The only method which has ever been used for the preparation of indolepyruvic acid is the hydrolysis of 2-phenyl-4-indolyl-methylene-5-oxazolone, a condensation product of 3-indolealdehyde with benzoylglycine (*2*).

Inaccessibility of 3-indolealdehyde in this method has decreased the availability of indolepyruvic acid, although this substance is of much biological interest.

In the author's method, the starting materials are methyl indoleacetate and diethyl oxalate as shown below.



With the progress of the so-called oxo-synthesis, it has come to be

possible to prepare methyl β-formylpropionate and concequently methyl indoleacetate in quantity (*3*).

The most difficult point of this method was the hydrolysis of methyl α-ethoxalylindoleacetate, because of the instability of indolepyruvic acid against mineral acid. This difficulty, however, was eliminated by heating with acetic acid in the presence of a small amount of paratoluenesulfonic acid. Under this treatment, methyl α-ethoxalylindoleacetate was converted directly to indolepyruvic acid with evolution of carbon dioxide.

In this paper, procedures are described for the preparation of indolepyruvic acid from methyl indoleacetate, and L-tryptophan from indolepyruvic acid by means of heart muscle transaminase.

EXPERIMENTAL

Preparation of Methyl α-Ethoxalylindoleacetate—50.5 g. of diethyl oxalate was added to the alcoholic solution of sodium ethoxide (7.9 g. of sodium was dissolved in 95 g. of absolute ethyl alcohol) with vigorous stirring. After 10 minutes, 60 g. of methyl indoleacetate (143–6°/0.075 mm. Hg.) was added and the reaction mixture was allowed to stand overnight. Ethyl alcohol was then removed from the reaction mixture and the residue was dissolved in 350 ml. of water. The solution was extracted twice with 100 ml. of ether (From the ether extract, 16 g. of methyl indoleacetate was recovered;, and the aqueous layer was acidified with 16 g. of acetic acid. The methyl α-ethoxalylindoleacetate separated as an heavy oil, which was removed by extracting the mixture with three 60 ml. portions of ether.

The combined ether solution was dried over anhydrous sodium sulfate and the ether was distilled off. The residue, mainly consisting of methyl α-ethoxalylindoleacetate, weighed 72 g.

As this substance failed to be crystallized, it was converted to methyl α-methoxalylindoleacetate as follows; 2 g. of methyl α-ethoxalylindoleacetate was dissolved in 7 g. of methyl alcohol and then 0.15 ml. of concentrated hydrochloric acid was added. The reaction mixture was heated in a water bath kept at 73–76° for 4.5 hours. After cooling, the crystal deposited was filtered, washed with a small amount of methyl alcohol and dried in air. The crystal weighed 1.3 g. The melting point was 113–5° when measured in the ordinary manner.

Anal. Calcd. for	$C_{14}H_{13}NO_5$ CH_3OH		
	C, 58.63;	H, 5.53;	N, 4.56
Found	C, 58.77	H, 5.57;	N, 4.77

The 2.4-dinitrophenylhydrazone of methyl α-methoxalylindoleacetate was obtained as follows: 0.2 g. of methyl α-methoxalylindoleacetate was added to 2,4-dinitrophenylhydrazine solution (0.2 g. of 2,4-dinitrophenylhydrazine and 0.8 g. of concentrated

sulfuric acid in 2.4 g. of methyl alcohol) kept at 37°.

After an hour, the hydrazone was separated and then recrystallized with 50 per cent methyl alcohol. The yield was 0.2 g. with m. p. 193–194°.

Anal. Calcd. for $C_{20}H_{17}N_5O_8$			
	C, 52.75;	H, 3.74;	N, 15.38
Found	C, 52.80;	H, 3.80;	N, 15.49

Preparation of Indolepyruvic Acid—A mixture of 20 g. of crude methyl α-ethoxalylindoleacetate, 140 g. of acetate acid and 1.4 g. of para-toluenesulfonic acid was heated in a flask fitted with a fractionating column at such a rate as to distill off only methyl and ethyl acetate formed. Heating was continued for 6 hours and the acetic acid was removed under diminished presure.

The residue was extracted with dilute sodium carbonate solution until most of the indolepyruvic acid was dissolved. The insoluble matter, which amounted to about 6 g., was removed from the alkaline solution and washed twice with 20 ml. of water. The combined alkaline solution, which amounted to about 90 g., was acidified with 3 *N* hydrochloric acid until the pH fell to 2.0. After cooling, the precipitate was filtered with suction, washed with a small amount of water and dried. It weighed 6.5 g. and showed m. p. 198–200°. The second crop (0.8 g.) with the same melting point was obtained from the mother liquor. Total yield was 7.2 g. (52 per cent based on the methyl indoleacetate consumed).

The pure indolepyruvic acid with m. p. 208–210° was obtained when a small amount of resinous matters was removed by recrystallization; 0.5 g. of the crude indolepyruvic acid was dissolved with 5 ml. of ethyl acetate. After removing a small amount of insoluble matter by filtration, 10 ml. of chloroform was added to the ethyl acetate solution. After 30 minutes, the precipitate was removed by centrifugation and then 10 ml. of chloroform was added to the supernatant. The mixture was allowed to stand overnight and the precipitate was collected on a Büchner funnel and dried. The yield was 0.1 g.

Anal. Calcd. for $C_{11}H_9NO_3$			
	C, 65.02;	H, 4.43,	N, 6.89
Found	C, 64.79;	H, 4.55;	N, 6.97

Preparation of Enzyme Solution—Enzyme solution was prepared as reported in the previous paper (*1*).

Preparation of L-*Tryptophan*—5.0 g. of indolepyruvic acid (m. p. 198–200°), 4.0 g. of L-aspartic acid and 1.2 g. of monosodium glutamate monohydrate were added to 200 ml. of water. 20 per cent aqueous ammonia solution was dropped into the mixture until the clear solution was obtained.

To this mixture 20 ml. of 0.2 *M* phosphate buffer (pH 7.6) and 225 ml. of the enzyme solution were added and then the total volume was adjusted to 550 ml. with water. The reaction mixture was placed in a 600 ml. Erlenmeyer flask at 38°. After 78 hours, the mixture was heated to 80° for 10 minutes and the insoluble matter

was filtered off. The clear filtrate was evaporated in vacuo to about 300 ml. and then the pH was adjusted to 5.0 with dilute hydrochloric acid. After removing the insoluble matter, the clear solution was passed through a column of permutit DR (2×30 cm.), and then the solution was followed by 600 ml. of 2 per cent ammonia solution. The effluent was concentrated in vacuo to dryness and the residue was washed with a small amount of methyl alcohol and dried. It weighed 2.3 g. with m. p. 260–265°. After one crystallization from 60 ml. of water, this product melted at 280–283° and weighed 1.2 g.

Anal. Calcd. for $C_{11}H_{12}N_2O_2$
C, 64.70, H, 5.88, N, 13.71
Found C, 64.54, H, 5.67, N, 13.51

Specific rotation of this product, $[\alpha]_D^{25°}$ showed −30.9° (210 mg. in 24 ml. of water, 2 dm., 25°). According to Rockland (*4*), specific rotation of L-tryptophan is −32.15° (2.071 g. of L-tryptophan in 100 ml. of water, 4 dm., 25°).

The author's best thanks are due to Prof. S. Akabori for his continued interest and advice, and also to Dr. T. Yoshida of Ajinomoto Co. who kindly supplied methyl β-formylpropionate.

SUMMARY

1. Indolepyruvic acid was prepared by hydrolysis of methyl ethoxalylindoleacetate, the condensation product of methyl indoleacetate with diethyl oxalate.

2. L-Tryptophan was obtained from indolepyruvic acid by heart muscle transaminase in an yield of 24 per cent based on the methyl indoleacetate consumed.

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BILE ACIDS AND STEROIDS

VIII. STUDIES ON HOG BILE ACIDS. (PART 3) SYNTHESIS OF PROGESTERONE AND DESOXYCORTICOSTERONE ACETATE FROM α-HYODESOXYCHOLIC ACID

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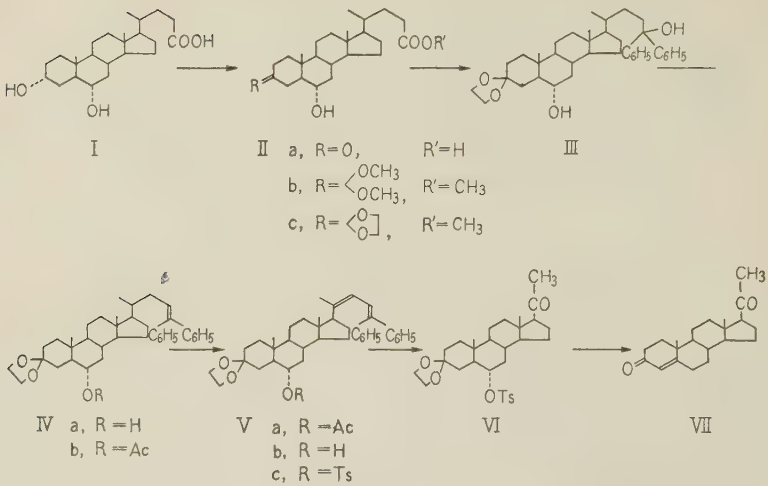
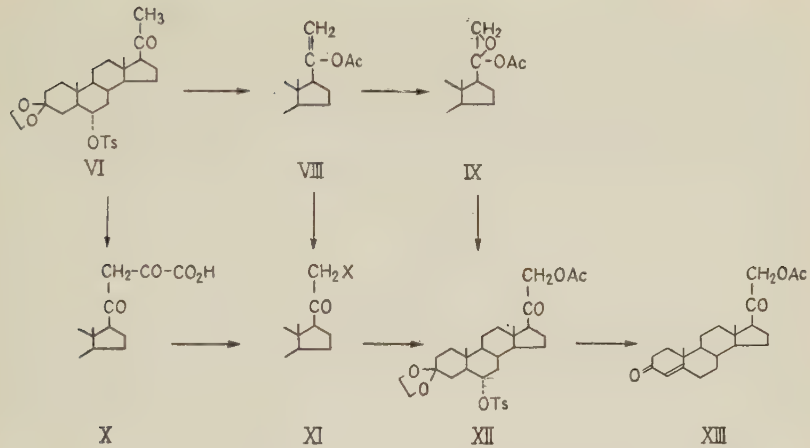
The previous paper in this series (*1*) has shown that α-hyodesoxycholic acid (I) is oxidized selectively at the C_3-hydroxyl group by treatment with *N*-bromosuccinimide in acetone-water and that 3-keto-6α-hydroxycholanic acid (IIa) is obtained. The present report describes the modification of the reaction of α-hyodesoxycholic acid with *N*-bromosuccinimide and the synthesis of progesterone (VII) and desoxycorticosterone acetate (XIII) by the degradation of 3-keto-6α-hydroxycholanic acid (IIa) under protecting the C_3-ketone with the ethylene ketal group.

Purification of 3-keto-6α-hydroxycholanic acid (IIa) has been difficult on account of the fact that IIa is more soluble than the starting material I, or that its methylester does not crystallize. For this reason it had been difficult and troublesome to determine the optimal oxidation condition of α-hyodesoxycholic acid (I) to IIa. In the present experiments, we found that crude 3-keto-6α-hydroxycholanic acid (IIa) was simultaneously esterified and ketalized by treatment with methanolic hydrochloric acid to give crystalline methyl 3,3-dimethoxy-6α-hydroxycholanate (IIb), and so a further investigation of the reaction conditions of I with *N*-bromosuccinimide could be carried out more easily. Thus this reaction was modified by change of acetone-water ratio and by addition of equivalent quantity of pyridine with *N*-bromosuccinimide. The oxidation was found to be completed by refluxing for several hours and the treatment of the product with methanolic hydrochloric acid gave methyl 3,3-dimethoxy-6α-hydroxycholanate (IIb) in a maximum yield of 64.5 per cent. Treatment of the dimethyl ketal IIb with ethylene glycol in benzene solution in the presence of *p*-toluenesulfonic acid afforded methyl 3-ethylenedioxy-6α-hydroxycholanate (IIc) in almost quantitative yield.



The diphenylcarbinol derivatives of several bile acids (*2, 3, 4*) have been prepared with a large excess of phenylmagnesium bromide at high temperature. The conversion of methyl 3-ethylenedioxy-6α-hydroxycholanate (IIc) into the corresponding diphenylcarbinol III was accomplished with 7 molar equivalent reagent at 0°, and the infrared spectrum of the crude product showed that the ethylene ketal group was entirely retained. The diphenylcarbinol III was dehydrated with iodine in benzene solution to the diphenylethylene IVa, while a small amount of a 3-keto compound by hydrolysis of the ethylene ketal group being formed. The diphenylethylene acetate IVb was brominated with *N*-bromosuccinimide in carbon tetrachloride solution followed by dehydrobromination with pyridine and alkaline hydrolysis to the diphenyldiene Vb.

The hydroxyl group at C_6 of Vb was converted into the tosylate for the purpose of detosylation to the 3-keto-Δ^4 group in distinction from the C_{21}-hydroxyl group of the ketol side chain, characteristic of desoxycorticosterone. The tosyloxy group was not affected by the conditions used in the reaction such as oxidation, enolization, hologenation and acetoxylation described below. The diphenyldiene tosylate Vc was oxidized with chromium trioxide in acetic acid at 0° to give 3-ethylenedioxy-6α-tosyloxypregnan-20-one (VI). Attempts to crystallize the oxidation product were unsuccessful, but the ultraviolet and



infrared absorption spectra supported the structure of VI.

Treatment of VI with potassium acetate in acetic acid caused simultaneously hydrolysis of the ethylene ketal group, detosylation and migration of the double dond thus formed, and afforded progesterone (VII).

The introduction of the ketol acetate side chain, characteristic of desoxycorticosterone acetate, into the pregnan-20-one containing the C_3-ethylene ketal group has been described by Sarett, *et al.* (*5*). Essentially according to their procedure 3-ethylenedioxy-6α-tosyloxy-21-acetoxypregnan-20-one (XII) was prepared by condensation with ethyl oxalate followed by iodination, ketonic cleavage and acetylation. The other feasible courses for preparation of the ketol acetate side chain under retaining the ethylene ketal group proceeded *via* the Δ^{20}-enol acetate involving either bromination followed by replacement of bromine with iodine and acetoxylation (*6, 7*) or peracid oxidation followed by acetoxyl rearrangement on silica gel (*8*). Both these routes afforded also the 20-keto-21-acetate XII described above.

Hydrolysis and detosylation of XII with potassium acetate in acetic acid as described for progesterone (VII) gave desoxycorticosterone acetate (XIII).

EXPERIMENTAL

All melting points were taken in capillary tubes, and are uncorrected. Infrared spectra were determined with a Perkin-Elmer Single-beam Infrared Spectrophotometer,

Model 12 C. The ultraviolet absorption spectra were measured in 95 per cent ethanol using a Beckman Model DU Spectrophotometer.

Methyl 3,3-Dimethoxy-6α-hydroxycholanate (*IIb*)—To a solution of 30 g. of α-hyodesoxycholic acid (I) and 8 g. of pyridine in 420 ml. of acetone and 180 ml. of water was added 17.7 g. of *N*-bromosuccinimide. The resulting mixture was refluxed for 5 hours, and then the solvent was removed under reduced pressure to the point of distinct turbidity. After a large volume of water was added, the precipitate was collected and dried.

This crude keto acid was dissolved in 135 ml. of hot methanol, cooled to room temperature and 15 ml. of 10 per cent methanolic hydrochloric acid was added. The mixture was allowed to stand at room temperature overnight, and the precipitated crystal was collected, 22 g. (64 per cent), plates, m. p. 153–157° (effervescence). After recrystallization from methanol and drying at 90° *in vacuo* for 5 hours the melting point was 160–162° (effervescence), $[\alpha]_D^{26}$ +2.9° (c=1.15, chloroform), no appreciable absorption in the ultraviolet spectrum.

Analysis.	Calcd. for $C_{27}H_{46}O_5$:	C 71.96,	H 10.29
	Found:	C 71.96,	H 10.41

Methyl 3-Ethylenedioxy-6α-hydroxycholanate (*IIc*)—A mixture of 30 g. of methyl 3,3-dimethoxy-6α-hydroxycholanate (IIb) and 0.45 g. of *p*-toluenesulfonic acid monohydrate in 600 ml. of benzene and 15 ml. of ethylene glycol was slowly distilled for 6 hours. More benzene was added dropwise to keep the volume above 500 ml. The cooled mixture was washed with sodium bicarbonate solution, then with water, and dried over sodium sulfate. The solution was concentrated until crystals began to separate. After cooling the crystals were collected, 27 g. (90 per cent), m. p. 194–197°. Concentration of the mother liquor gave an additional 2.2 g. (7.3 per cent) of IIc, m. p. 186–191°. Pure IIc (from ethyl acetate), plates, m. p. 194–197°, $[\alpha]_D^{20}$ +1.33° (c=1.05, chloroform).

Analysis.	Calcd. for $C_{27}H_{44}O_5$:	C 72.28,	H 9.88
	Found:	C 72.10,	H 9.99

3-Ethylenedioxy-24,24-diphenyl-Δ^{23}-cholen-6α-ol (*IVa*)—A solution of 20 g. of methyl 3-ethylenedioxy-6α-hydroxycholanate (IIc) in 680 ml. of dry benzene was added dropwise with stirring at 0° during 2 hours to a solution of phenylmagnesium bromide prepared from 8.1 g. of magnesium, 55 g. of bromobenzene and 220 ml. of anhydrous ether. After the complete addition of the benzene solution, stirring was continued for 1 hour, the mixture was decomposed with saturated ammonium chloride solution. The organic layer was washed with water, and dried over sodium sulfate. To the solution was added 0.1 g. of iodine, and the solvent was distilled off at which time the diphenylcarbinol III was dehydrated to the diphenylethylene IVa. The residue was heated under reflux for 1 hour with a mixture of 6 g. of potassium hydroxide and 300 ml. of methanol to saponify any unreacted ester, and then steam distilled until the biphenyl was completely removed. The residual solid was extracted with benzene,

washed with water, dried over sodium sulfate and concentrated. The residue was crystallized from ethyl acetate to give 16.9 g. (68.5 per cent), rods, m. p. 182–187°.

After concentrating the mother liquor, the residue was reketalized with ethylene glycol and *p*-toluenesulfonic acid in benzene by the usual method. There was thus obtained an additional 4.4 g. (17.8 per cent) of IVa, m. p. 184–188°.

Recrystallization from ethyl acetate gave material with m. p. 188–190°, $[\alpha]_D^{23}$ +38.0° (c=0.97, chloroform), λ_{max}^{EtOH} 252 mμ (log ε 4.22).

Analysis. Calcd. for $C_{38}H_{50}O_2$: C 82.26, H 9.08
Found: C 82.59, H 9.11

3-Ethylenedioxy-24,24-diphenyl-Δ^{23}-cholen-6α-ol Acetate (*IVb*)—To a solution of 4.9 g. of the above cholen-6α-ol IVa in 30 ml. of pyridine was added 15 ml. of acetic anhydride. The mixture was allowed to stand at room temperature overnight. To the chilled solution was added a small amount of water, and after a short time the solution was diluted with benzene and with water. The benzene extract was washed with dilute acid, dilute alkali, and with water, and dried over sodium sulfate. After removal of the solvent under reduced pressure the product was crystallized from ether and 4.9 g. (92.8 per cent), m. p. 102–104° were obtained in two crops. For analysis it was recrystallized from acetone, m. p. 103–105°, $[\alpha]_D^{27}$ +44.6° (c=0.825, chloroform).

Analysis. Calcd. for $C_{40}H_{52}O_2$: C 80.50, H 8.78
Found: C 80.09, H 8.85

3-Ethylenedioxy-24,24-diphenyl-$\Delta^{20(22),23}$-choladien-6α-ol (*Vb*)—A mixture of 5.25 g. of the cholene acetate IVb and 1.60 g. of *N*-bromosuccinimide in 80 ml. of carbon tetrachloride was refluxed for 15 minutes under illumination with a 500 watt reflector spot light. After cooling, precipitated succinimide was removed by filtration. To this solution was added 5 ml. of pyridine, carbon tetrachloride was distilled off, and the residue was heated on the steam bath for an additional hour. After cooling, dilute hydrochloric acid was added and the mixture was extracted with ether. The extract was washed with water, and dried over sodium sulfate. The solvent was removed and the residue was hydrolyzed by refluxing in 150 ml. of 3 per cent alcoholic potassium hydroxide for 2 hours. The mixture was concentrated under a reduced pressure, diluted with water and extracted with benzene. The benzene extract was washed with water, dried and concentrated. The residue was crystallized from acetone giving needles, 2.8 g., m. p. 186–188°. The mother liquor gave an additional 0.6 g., m. p. 169–174°, (total 69.8 per cent). After recrystallization of the first crop an analytical sample was obtained, m. p. 188–190°, which was depressed to 169–177° on admixture with the cholen-6α-ol IVa of m. p. 188–190°. $[\alpha]_D^{12}$ +41.8° (c=0.95, chloroform), λ_{max}^{EtOH} 306 mμ (log ε 4.44).

Analysis. Calcd. for $C_{38}H_{48}O_3$: C 82.57, H 8.75
Found: C 82.52, H 8.77

3-Ethylenedioxy-24,24-diphenyl-$\Delta^{20(22),23}$-choladien-6α-ol Tosylate (*Vc*)—To a solution

of 5.6 g. of the crude crystalline Vb in 23 ml. of pyridine was dissolved 5.6 g. of *p*-toluenesulfonyl chloride, and the mixture was allowed to stand at room temperature overnight. A small volume of water was added to the chilled solution and after a short time the solution was diluted with benzene and with water. The benzene layer was washed with dilute acid, dilute sodium carbonate solution, and with water, and dried over sodium sulfate. The solvent was removed and the residue was crystallized from ether to give 5.6 g. (78.1 per cent) of prisms, m. p. 180–182° (decomp.). Recrystallization from acetone afforded an analytical sample with m. p. 181–182° (decomp.), $[\alpha]_D^{26}$ +28.9° (c=1.085, chloroform), λ_{max}^{EtOH} 225, 306 mμ (log ε 4.40, 4.45).

Analysis. Calcd. for $C_{45}H_{54}O_5S$:	C 76.45,	H 7.70
Found:	C 76.08,	H 7.62

3-Ethylenedioxy-6α-tosyloxypregnan-20-one (*VI*)—A solution of 5.0 g. of the choladiene tosylate Vc in 75 ml. of ethylene chloride and 75 ml. of 90 per cent acetic acid was cooled to −10°, and 75 ml. of 90 per cent acetic acid containing 3.5 g. of chromium trioxide was added dropwise with stirring during 1 hour. After standing 20 hours at 0°, the excess oxidizing agent was destroyed with sulfur dioxide under chilling to −10°. The mixture was poured into 500 ml. of cold water and extracted with 250 ml. of ether. The extract was washed with 5 per cent sodium carbonate solution, then with water and dried over sodium sulfate. Removal of the solvent left 5.3 g. of oily residue, which was chromatographed on 60 g. of silica gel.

The first fraction eluted with petroleum ether-benzene (1:1 to 1:3) gave 1.3 g. of the counterpart of the aimed product, diphenylacroleine.

The second fraction eluted with petroleum ether-ether (2:1 to 1:1) yielded 2.4 g. of viscous oil. Attempts to crystallize this product were unsuccessful. The structure was verified as 3-ethylenedioxy-6α-tosyloxypregnan-20-one (VI) by its infrared absorption data; 20-carbonyl, 5.88 μ; ethylene ketal, 9.12 μ; tosyloxy, 7.37, 8.41 and 8.50 μ. Thus this substance was used directly for the reaction described below.

Further elution with ether gave 1.2 g. of glassy oil, which was not obtained in crystalline form. The infrared absorption spectrum showed characteristic absorption for 3-carbonyl (5.86 μ), but none for ethylene ketal, then the structure of this substance was presumed to be 6α-tosyloxypregnane-3,20-dione, which was formed by hydrolysis of the ethylene ketal group with acetic acid during the oxidation reaction.

Progesterone (*VII*)—A mixture of 1.0 g. of oily 3-ethylenedioxy-6α-tosyloxypregnan-20-one (VI) and 6 g. of potassium acetate in 30 ml. of glacial acetic acid was refluxed for 2 hours. The reaction mixture was poured into water, and taken up in benzene. The benzene extract was washed with sodium carbonate solution, then with water, and dried. The benzene was removed *in vacuo*. Crystallization of the resulting residue from ether-hexane furnished 0.28 g., m. p. 119–122°. The additional 0.12 g. of VII was obtained from the mother liquor through chromatography on alumina. Total yield 0.40 g. (67.5 per cent). Recrystallization from aqueous alcohol raised the melting point to 127–129°; no depression by admixture with an authentic specimen. λ_{max}^{EtOH} 241 mμ (log ε 4.15).

Analysis. Calcd. for $C_{21}H_{30}O_2$: C 80.21, H 9.62
Found: C 80.42, H 9.80

Similar treatment of 1.0 g. of 6α-tosyloxypregnane-3,20-dione gave also 0.41 g. of progesterone (VII), m. p. 119–122°; no depression on admixture with an authentic sample.

3-Ethylenedioxy-6α-tosyloxypregnan-20-one-21-oxalyl Acid (*X*)—To a solution of dry sodium ethoxide prepared from 0.08 g. of sodium and 1.5 ml. of absolute alcohol, 0.56 g. of ethyl oxalate and 4 ml. of anhydrous benzene was added 0.50 g. of 3-ethylenedioxy-6α-tosyloxypregnan-20-one (VI). The homogeneous yellow solution was allowed to stand at room temperature overnight and poured into a mixture of ice and phosphoric acid. The mixture was extracted several times with ether, and the combined ether solution was shaken with sodium hydroxide solution. The resulting precipitate of the sodium enolate slowly dissolved by standing overnight into the alkaline layer, which was acidified with phosphoric acid under cooling and extracted three times with ether. The combined ether extract was washed with water and dried. Evaporation of the ether afforded yellow oil, 0.58 g. An attempt to crystallize this product was unsuccessful, but the substance gave a positive ferric chloride reaction and the infrared spectrum confirmed the given structure.

3-Ethylenedioxy-6α-tosyloxy-Δ^{20}-pregnen-20-ol Acetate (*VIII*)—A solution of 2.0 g. of the foregoing oily substance VI in 40 ml. of isopropenyl acetate containing 0.2 g. of *p*-toluenesulfonic acid monohydrate was slowly distilled for 14 hours. More isopropenyl acetate was added dropwise to keep the initial volume. The reaction mixture was cooled and after dilution with water it was extracted with ether. The ether extract was washed with sodium bicarbonate solution, then with water and dried over sodium sulfate, and the solvent was removed under a reduced pressure. The brownish viscous residue was dissolved in benzene, and the solution was passed through a short column of alumina. Concentration of the filtrate left 2.1 g. of colourless glassy oil, which was not obtained in crystalline form. The infrared spectrum showed characteristic absorption for Δ^{20}-enol acetate (5.72, 5.99 and 8.18 μ), tosyloxy (7.37, 8.40 and 8.51 μ) and ethylene ketal (9.12 μ), and a slight absorption of 20-carbonyl (5.88 μ) due to the starting material remaining. This product was used directly for the next step described below without purification.

3-Ethylenedioxy-6α-tosyloxy-21-acetoxypregnan-20-one (*XII*). (*a*) *From the 21-Oxalyl Acid X*—The crude 21-oxalyl acid X described above (0.58 g.) was dissolved in 150 ml. of water containing 6.6 g. of disodium hydrogen phosphate, and a solution of 2.4 g. of iodine in 25 ml. of ether was added dropwise with stirring at room temperature during 45 minutes. After the addition was completed, a solution of 0.33 g. of potassium hydroxide in 20 ml. of water was added and the reaction mixture was stored in the refrigerator overnight at which time a yellow precipitate appeared. The resulting mixture was taken up in ethyl acetate, and the extract was washed with water and dried. Removal of the solvent *in vacuo* gave 0.45 g. of the crude oily iodo ketone, which gave a positive Beilstein's reaction and a negative ferric reaction. This substance was dissolved in 40 ml. of acetone, and refluxed for 20 hours with 4 g. of potassium bicarbo-

nate and 2.35 g. of glacial acetic acid, then diluted with water and extracted with benzene. The benzene solution was washed with water, dried over sodium sulfate and the solvent was removed *in vacuo*. The residual oil was dissolved in benzene, and filtered through a short alumina column. Evaporation of the filtrate gave a colourless oil, which was crystallized from acetone-hexane yielding 0.09 g. of needles, m. p. 177–179° (decomp.). Recrystallization from acetone-hexane afforded the anticipated material XII with m. p. 179–180° (decomp.), $[\alpha]_D^{21}$ +39.25° (c=0.94, acetone), $\lambda_{max}^{CS_2}$ 5.71 and 5.78 μ (21-acetoxy-20-keto); 8.42 and 8.50 μ (tosyloxy); 9.12 μ (ethylene ketal).

Analysis. Calcd. for $C_{32}H_{44}O_8S$: C 65.29 H 7.53
Found: C 65.27, H 7.65

(*b*) *From the Enol Acetate VIII via the 21-Bromo Ketone XI*: A solution of 0.5 g. of the crude enol acetate VIII described above in 10 ml. of methylene chloride was cooled below −10° in an ice-salt bath. To this, 8 ml. of 0.0875 *M* bromine solution in methylene chloride (about 80 per cent of theoretical amount) was added dropwise with stirring until the bromine colour was persistent for 5 minutes. The resulting solution was diluted with ether and washed with cold 1 per cent sodium hydroxide solution, then with water and dried over sodium sulfate. The solvent was removed under a diminished pressure. This crude bromo compound was allowed to stand at room temperature for 30 minutes in 50 ml. of acetone with 0.5 g. of sodium iodide. The precipitated sodium bromide was filtered and the filtrate was refluxed for 16 hours with a mixture of 5.0 g. of potassium bicarbonate and 3.0 g. of glacial acetic acid. After concentration and dilution with water, the product was extracted with benzene, washed with water, dried and evaporated. The residue was chromatographed on silica gel, and the fraction eluted with benzene-ether (5:1) was crystallized from acetone-hexane to give 0.13 g. of crystals with m. p. 162–166° (decomp.). Recrystallization from the same solvent raised the melting point to 178–180° (decomp.), no depression upon admixture with a sample prepared by the above mentioned method (a).

Analysis. Calcd. for $C_{32}H_{44}O_8S$: C 65.29, H 7.53
Found: C 65.39, H 7.60

(*c*) *From the Enol Acetate VIII via the Epoxide IX*—A mixture of 0.75 g. of the crude enol acetate VIII and 22 ml. of 1.178 *M* perbenzoic acid solution in benzene was allowed to stand at room temperature for 24 hours. The reaction mixture was diluted with ether and was extracted with dilute sodium hydroxide solution and with water. The organic layer was dried over sodium sulfate, and the solvent was removed *in vacuo*. The oily residue was dissolved in petroleum ether-benzene (1:3) and was adsorbed on silica gel column and allowed to stand overnight. The benzene-ether (4:1) eluate gave a glassy oil which crystallized from acetone-hexane, yield 0.08 g., needles, m. p. 174–177° (decomp.). Pure XII showed m. p. 178–180° (decomp.) (from acetone-hexane), identical with an authentic specimen prepared by method (a).

Desoxycorticosterone Acetate (*XIII*)—A mixture of 100 mg. of the 20-keto-21-acetate

XII and 1 g. of potassium acetate in 5 ml. of glacial acetic acid was heated under reflux for 1 hour. After cooling the reaction mixture was diluted with water and with benzene, and the benzene layer was washed with sodium carbonate solution, then with water and dried over sodium sulfate. The solvent was removed *in vacuo* and the residue was crystallized from ether-hexane to give 33 mg. of needles, m .p. 147–151°. Chromatographic purification on alumina of the mother liquor afforded an additional 10 mg., m. p. 148–151°. Total yield 43 mg. (68 per cent). After recrystallization from acetone-hexane the substance melted at 154–156°, and identity with an authentic specimen was established by a mixed melting point determination and by comparison of the infrared spectra.

Analysis. Calcd. for $C_{23}H_{32}O_4$:	C 74.16,	H 8.66
Found:	C 74.47,	H 8.78

SUMMARY

The procedure for the oxidation of α-hyodesoxycholic acid (I) with *N*-bromosuccinimide to 3-keto-6α-hydroxycholanic acid (IIa) was reinvestigated and modified. The degradation of II by the Miescher method proceeded smoothly under protecting the C_3-ketone by introducing the ethylene ketal group. The C_6-hydroxyl group was converted to the tosylate in order to facilitate its elimination, and 3-ethylenedioxy-6α-tosyloxypregnan-20-one (VI) was obtained. Introduction of the C_{21}-acetoxyl group leading to 3-ethylenedioxy-6α-tosyloxy-21-acetoxypregnan-20-one (XII) was accomplished using both routes, *via* the 21-oxalyl acid X and *via* the Δ^{20}-enol acetate VIII. Treatment of VI and XII with potassium acetate in acetic acid gave progesterone (VII) and desoxycorticosterone acetate (XIII), respectively.

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SOLUBILIZATION OF THE PARTICULATE HYDROGENASE

Sirs:

It is common knowledge that a great many species of bacteria, strict anaerobes, facultative anaerobes, and even strict aerobes such as *Azotobacter vinelandii* exhibit hydrogenase activity. The enzyme activity of bacterial extracts, however, is almost localized in the insoluble particle fraction, except that of *Clostridium* type. Consequently it seemed that the hydrogenase of strict anaerobes such as *Clostridium* is contained in a soluble fraction and of other sources is contained in insoluble particles. Nevertheless, in the case of *Desulfovibrio desulfuricans*, a member of strict anderobes, about 90 per cent of the total activity of the extract is localized in the particulate fraction, *i.e.* the precipitate obtained under the centrifugal field at 100,000$\times$g. for 2 hours.

For the purification of this enzyme, it is necessary to solubilize the enzyme protein. We have succeeded to solubilize the particulate hydrogenase with the following method:

1) Wet cells are suspended in 3–4 volumes of 3–4 per cent solution of sodium desoxycholate and disrupted by ultrasonic oscillation (560 KC) for 15 minutes.

2) Cell debris are removed by centrifuging at 3,000$\times$g. for 15 minutes.

3) The supernatants (S) is diluted with 2–3 volumes of $M/150$ phosphate buffer (pH 7.0), then adjusted to pH 8.0 with N-NaOH solution, and finally crystalline trypsin (about 1 per cent of total protein in the supernatant) is dissolved in the supernatant. It is incubated at 37° for one hour.

4) The trypsin-digest (D) is adjusted to pH 7.0. Saturated solution of $(NH_4)_2SO_4$ is added to it up to 0.3 saturation. The precipitate is removed by centrifugation.

5) Saturated solution of $(NH_4)_2SO_4$ is added to the supernatant up to 0.6 saturation, and then the enzyme protein is precipitated (P).

The precipitate can be dissolved in $M/150$ phosphate buffer (pH 7.0) and no precipitates appear by centrifugation at 100,000$\times$g. for 2 hours.

Total and specific activities are shown in Table I.

TABLE I

	Total activity (recovery %)	Specific activity (μl H_2/hr./prot. N mg.)
(S)	100	20,000
(D)	70	56,000
(P)	50	340,000

Assay method: H_2 evolution from reduced methylviologen (*1*, *2*). For (S), (D) and (P), see the text.

In the original extract the enzyme protein precipitates by adding $(NH_4)_2SO_4$ into it up to 0.3 saturation. When only desoxycholate has been added, the enzyme protein is not precipitated by centrifugation at $100,000 \times g$., but is precipitated at 0.3 saturation of $(NH_4)_2SO_4$. In this concentration of $(NH_4)_2SO_4$ desoxycholate is precipitated. When the original extract has been digested in the absence of desoxycholate the yields of the soluble enzyme is lower than the above mentioned.

Hydrogenase of other microorganisms can also be solubilized by this method. The yields of the soluble hydrogenase are shown in Table II.

TABLE II

	D	DT
Escherichia coli	44	95
Proteus vulgaris	—	86

The figures in this table represent percent recovery against the activity of the original extract obtained by centrifugation at $2,000 \times g$ for 20 minutes. D: Supernatant obtained by centrifugation at $100,000 \times g$ for 2 hours after ultrasonic disruption of bacterial cells in the presence of desoxycholate. DT: Supernatant obtained by centrifugation at $100,000 \times g$ for 2 hours after trypsin-digestion of the desoxycholate-extract.

The present procedures is now being applied to purify the enzymes obtained from various sources and to compare their properties.

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ÜBER DIE CHROMATOGRAPHISCHE TRENNUNG VON KONDENSIERTEN PHOSPHATENAN ANIONENAUSTAUSCHERHARZEN

Die Trennung von niederen kondensierten Phosphate verschiedener Kondensationsgrade wurde wesentlich erst durch die von Ebel (*1*) angefangenen papierchromatographischen Methode ermöglicht. Grunze und Thilo (*2*) haben diese Methode so entwickelt, daß eine Trennung bis zum oktameren, unter günstigen Umstände bis zum dekameren Polyphosphate auf dem Papier möglich wurde (*3*). Anderseits hat die hauptsächlich von Bauenkamp *et al.* (*4*) bearbeitete anionenaustauschchromatographische Methode schon auf dem Gebiete der Biochemie ihre wichtige Rolle gezeigt (*5*).

Die chromatographische Trennung der kondensierten Phosphate an Anionenaustauscher wurde bei mir weiter studiert und es ist gelungen, durch lineare Steigerung der KCl Konzentration in der Elutionsflüssigkeit eine feine Trennung der kettenförmigen kondensierten Phosphate reihenweise von Mono- bis etwa Dodekameren zu erreichen.

Für die Probesubstanz des Polyphosphategemisches ab Mono- bis Hochpolymeren wurde handelsübliches Grahamsches Salz (*6*) verwendet, die durch Bariumfällung von Tri- und Tetrametaphosphaten befreit und nach Entkationieren durch Amberlite IR-120 mit Ammoniak neutralisiert wurde. Die Anionenaustauschersäule von Dowex 1-4X, Cl^--Form und 200–400 Maschen, 1.1 cm. von Durchmesser und 30 cm. hoch wurde mit 10 Volumen Pufferlösung von 0.005 *M* Borax-Borsäure von pH 8.0 bzw. 0.01 *M* Ammoniak-Ammonchlorid von pH 9.3 gewaschen. Zwei ml. der Probelösung, die etwa 1 mg. je Komponente enthielt, wurde darauf absorbiert und mit den obige Puffersubstanzen enthaltenden Elutionsflüssigkeiten, in denen die KCl Konzentration linear in 2 L. von 0.2 *M* zu 0.5 *M* aufsteigen sollte, ablaufen lassen. Der dabei gebrauchte Apparat bestand aus zwei mit Glasrohr aneinander verbundenen gleichen Gefässe, wie in Abb. 1 gezeigt wird: das erste enthielt 1 L. der 0.2 *M* KCl Lösung, das zweite 1 L. der 0.5 *M* KCl Lösung, die beiden Lösungen mit 0.005 *M* Borax-Borsäure zu pH 8.0 bzw. mit 0.01 *M* Ammoniak-Ammonchlorid zu pH 9.3 pufferisiert waren. Die Lösung floss vom ersten Gefäss durch die Säule hinunter und wurde mit dem Fraktionssammler in Portionen von je 9.0 ml. aufgefangen und das mit 2 *N* Schwefelsäure bei 100° in 10 Minuten hydro-

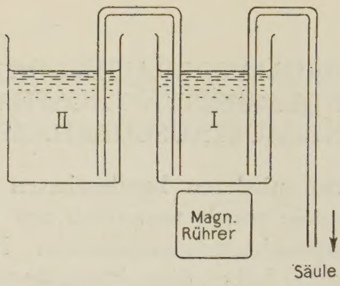

ABB. 1. Vorrichtung zur Kontinuierlichen Änderung der Elutionsflüssigkeit.

lysierbare Phosphat wurde gemessen.

Die Trennung war, wie aus Abb. 2 ersichtlich, bis zum Heptameren ganz einwandfrei und die noch höheren Polyphosphate waren mindestens bis zum Dodekameren eindeutig nachzuweisen. Tri- und Tetrametaphosphate (*7*) verhielten sich unter diesen Bedingungen ganz gleich aber an Dowex 1–8X trennten sich die beiden klar voneinander (Abb. 2,3). Die Chromatographie verlief bei bestimmter Temperatur mit sehr sicherer Reproduzierbarkeit. Mit dieser Methode erschien eine neue

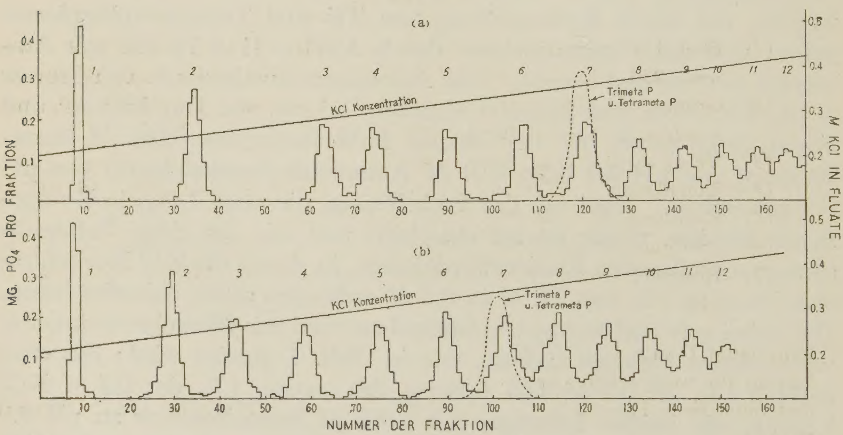

ABB. 2. Trennung der kondensierten Phosphate an Dowex 1–4X. (a): Boraxpuffer, pH 8.0. (b): Ammoniakpuffer, pH 9.3. Ablaufgeschwindigkeit: 1.2–1.8 ml./Min. Temperatur: 28°–32°.

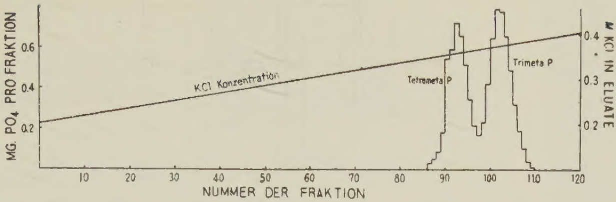

ABB. 3. Trennung der Trimeta- und Tetrametaphosphate an Dowex 1–8X.

Säule: 22 cm. × 1.1 cm.

Elutionsflüssigkeit: KCl Konzentration wie im Bild gezeigt, 0.005 *M* Boraxpuffer von pH 8.0.

Ablaufgeschwindigkeit: 1.8 ml./Min.

Temperatur: 25°–28°.

Probesubstanzen: Na-Trimeta P und Na-Tetrameta P, 0.05 mM je Komponente.

Möglichkeit, die einzelnen kondensierten Phosphate niederer als etwa Dekameren einfach und rein herzustellen und anderseits solche Phosphate in Natursubstanze wie Hefeextrakt zu identifizieren. Über ihre Anwendungsbeispiele wird später berichtet werden.

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